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E. FARKAS, J. HORVÁTH, S. KOTLÁN, R. MANNINGER,
A. PELC, K. RAUSS, J. SZIRMAI, J. WEISSFEILER

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MICROBIOLOGICAL DETERMINATION OF SMALL AMOUNTS OF CHLORIDE

By

J. KOLLÁR and M. JÁRAI

Biochemical and Microbiological Laboratories, Department of Antibiotics,
"Chinoin" Chemical & Pharmaceutical Works, Budapest

(Received July 1, 1959)

Summary. A quantitative method for the microbiological determination of small amounts of chloride has been developed on the basis that some *Streptomyces aureofaciens* strains are able to utilize exhaustively the chloride content of the medium for the synthesis of chlortetracycline. Determining the quantity of chlortetracycline synthesized by the system, the original concentration of chloride can be calculated.

At the beginning of our experiments concerned with the biochemical investigation of chlortetracycline (CTC) synthesis, calculation of experimental results was strongly hindered by the lack of a method suitable for the determination of the sometimes sufficiently low chloride concentration of the media used. None of the known methods [1—5] examined proved to be satisfactory for this purpose.

The observation of DOERSCHUK *et al.* [6] that some *Streptomyces aureofaciens* strains are able to utilize almost completely the chloride content of the medium suggested the use of this property for the selective estimation of low chloride concentrations.

Materials and methods

The CTC producing *Streptomyces aureofaciens* strain B—28, originating from the Soviet strain LS—536 [7], was used. According to our earlier observations, strain B—28 had the property to utilize exhaustively the chloride content of the medium and was therefore regarded as a so-called "total chloride utilizer".

Experiments were carried out with 100 ml media in 500 ml Erlenmeyer flasks. Spores were inoculated into a medium consisting of starch 2%; pea-nut meal, 2%; CaCO_3 , 0.4%; corn-steep liquor, 0.4%; palm oil 0.2%; pH 6.6—6.8. The inoculated flasks were incubated for 48 hr on an excentric shaker (220 rpm) at 27° C. After the incubation period, mycelia were centrifuged and washed three times with sterile distilled water. Aqueous suspensions of the washed mycelia, at the rate of 5 per cent, were used to inoculate synthetic media of the following composition: sucrose, 25 g; NH_4NO_3 , 5 g; KNO_3 , 3 g; CaCO_3 , 4 g; $\text{NH}_4\text{H}_2\text{PO}_4$, 100 mg; MgSO_4 , 100 mg; l-methionine, 100 mg; l-histidine, 50 mg; l-phenylalanine, 50 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 2 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 mg; thrice glass distilled water, 1000 ml; pH 6.5. All the materials used were of analytical grade. Mixing 1 ml of the sterilized medium with 3 drops of reagent HNO_3 and 1 ml of 10 per cent AgNO_3 , no opalescence resulted in ten minutes. The above synthetic medium were used for determination of the exact rate of chloride utilization and to construct a standard curve.

The butanolic extracts of the fermentation broths were assayed for total antibiotics content by the ferrichloride colour reaction. Spectrophotometric measurements were carried out with a Beckman Model B spectrophotometer. Comparative tests showed a difference of only 3 per cent between the colour intensity of the ferric complexes of CTC, and TC (tetra-

cycline) therefore the data were calculated on the basis of a common standard curve plotted from the average values of standard curves prepared with CTC and TC, respectively. Accuracy of the method was found to be ± 2.2 per cent.

To measure the relative amounts of CTC and TC, the paper chromatographic method of FISCHBACH and LEVINE [8] was applied with some modifications. The acid filtrate of the fermentation broth was extracted with n-butanol in the presence of 20 per cent sodium chloride and the butanolic layer was separated by centrifugation. An aliquot of the butanolic solution containing 1.0–1.5 mg antibiotics was placed on the paper (Schleicher & Schüll 2040 a) and developed with the water saturated mixture (v/v) of methylisobutylketone: butylacetate: isobutanol (15:5:2). Two parts of formic acid were added to the mixture before use. Development was allowed to proceed until the solvent front had travelled 12–13 cm. This usually took 40–45 min. The strips were then air dried and the antibiotics spots located by their fluorescence in ultraviolet light after 1.5 min treatment with ammonia vapours. Spots of CTC and TC were cut off and eluted with ice cold phosphate buffer (pH 4.5). For assay of the antibiotic activity of CTC and TC, respectively, the microbiological agar-diffusion method was applied, using *B. subtilis* strain ATCC—6633 as test organism. Chromatograms from each experimental flask were run in triplicate and each of the eluates was tested on four plates. The standard deviation of the combined method did not exceed ± 10 per cent; it was found capable of detecting as little as 3–4 per cent of CTC beside 96–97 per cent of TC.

Results

A series of experimental flasks were inoculated and sodium chloride was added in various concentrations (0.1–1.5 mM). Each level of sodium chloride was examined in four parallel flasks. Total antibiotic concentration and distribution of individual antibiotics in the fermentation broth were determined after an appropriate cultivation period. The results of a typical experiment are illustrated in Table I.

Table I

Synthesis of CTC in the presence of various chloride concentrations

Chloride conc. mM	Total antibiot. conc. $\mu\text{g}/\text{mg}$	CTC determined experimentally		CTC calculated theoretically on chloride conc. $\mu\text{g}/\text{ml}$	Percentage recovery of chloride in CTC
		$\mu\text{g}/\text{ml}$	%		
0.10	755	45	6.0	48	93.7
0.25	755	118	15.6	120	98.3
0.50	750	245	32.4	240	102.1
0.75	755	356	47.1	360	98.8
1.00	755	475	63.0	479	99.3
1.20	745	570	75.5	575	99.0
1.40	765	666	88.2	671	99.3
1.50	755	686	91.4	719	95.4
1.60	760	711	94.1	767	92.7

It is seen from Table I that the amount of CTC synthesized is fairly proportional to the chloride concentration of the medium and, except the

last values, chloride utilization may be regarded to be complete. This connection is shown graphically in *Fig. 1*, expressed in milliequivalents.

Fig. 1 as well as the data of *Table I* show that within suitable concentration ranges synthesis of CTC is a linear function of the chloride concentration. Similar connections between CTC and chloride concentrations were obtained in other experiments, where values for total antibiotic production varied from 450 $\mu\text{g/ml}$ to 1080 $\mu\text{g/ml}$.

These results indicate that the method is utilizable for the determination of chloride. The chloride concentration of a given medium can be calculated

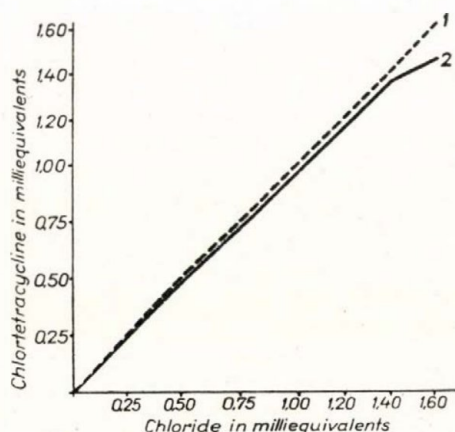


Fig. 1. Synthesis of CTC by strain B-28 on synthetic medium. 1: Synthesis of CTC in the case of theoretical chloride utilization. 2: Synthesis curve found experimentally.

on molar basis from the experimentally estimated values of CTC. The following equation may be used for calculation:

$$X = \frac{C}{13.5}$$

where X is the concentration of chloride, while C the experimentally determined CTC concentration in $\mu\text{g/ml}$. It should be noted that with high CTC concentrations, when CTC exceeds 90 per cent, the determination must be repeated using an appropriate dilution.

Similar results obtained with strain B-46 of the same origin (also a "total chloride利用者"), allows the general conclusion that all *Streptomyces aureofaciens* strains belonging to the first class of DOERSCHUK *et al.* [6] are suitable for the microbiological determination of chloride.

The usefulness of the method was controlled by adding known amounts of sodium chloride to complex media and measuring the recovery. The results of such an experiment are summarized in *Table II*.

Table II
*Recovery of chloride on complex medium**

NaCl added μg/ml	CTC formed μg/ml	Chloride found μg/ml	Percentage recovery
—	118	8.0	—
5	168	12.5	96.1
15	310	22.9	99.7
45	702	51.9	98.0

* Total antibiotic production in this experiments was on the average 1280 μg/ml.

The results are not influenced by the presence of equivalent amounts of halide homologues. Beside the common culture media used for *Streptomyces aureofaciens*, the method is applicable for determination of the chloride content of any aqueous solution which does not contain toxic substances. In this case an aliquot of the solution to be tested is added to the above synthetic medium or any other medium of known chloride content suitable for the cultivation of the used strain. The medium is inoculated with the strain and after appropriate incubation the CTC formed is estimated. Though the method is undoubtedly time consuming, it offers the advantage of allowing determination of low — otherwise hardly and uncertainly estimable — chloride concentrations. Since there was no more than 20 per cent deviation between the extreme values of our experiments, the accuracy of the method can be given as ± 10 per cent.

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Address of the authors: GYULA KOLLÁR, MIKLÓS JÁRAI
Biochemical and Microbiological Laboratories, Department of Antibiotics,
"Chinoïn" Chemical and Pharmaceutical Works, Budapest IV. Tó u. 1/5.

BIOCHEMICAL STUDIES ON STREPTOMYCES AUREOFACIENS

I. STUDIES ON THE CHLORINATION MECHANISM

By

J. KOLLÁR and M. JÁRAI

*Biochemical and Microbiological Laboratories, Department of Antibiotics,
"Chinoin" Chemical & Pharmaceutical Works, Budapest*

(Received July 1, 1959)

Summary. (i) The effect of chloride ions on the biosynthesis of chlortetracycline has been investigated and the conclusion was reached that chlorination occurs at a relatively early stage of chlortetracycline synthesis.

(ii) The results of experiments carried out with various halide homologues supported the earlier statement, regarding the competitive inhibition of chlorination by these anions.

(iii) Experimental findings obtained with copper and compounds inhibiting chlorination support the view that copper is taking some part in this process.

The chlorination process in the biosynthesis of the tetracyclines has been investigated by several workers, and in the course of their experiments bromide ions [1], halide homologues and various thiocompounds [2-5], as well as chloride concentration [6] were examined regarding their effect on the formation of chlortetracycline (CTC).

Beside the re-examination of the previous results the present paper is concerned with detailed investigations of the CTC producing strain, *Streptomyces aureofaciens*. In the course of these experiments data have been obtained concerning nature and time course of the chlorination reaction.

Materials and methods

Shaken cultures of the CTC producing *Streptomyces aureofaciens* strain B-28 [7] were used. The methods employed were described previously [7] with the only exception that a complex fermentation medium of the following composition was used: starch 3%; pea-nut meal 0.6%; NH_4NO_3 0.6%; CaCO_3 0.5%; KH_2PO_4 0.02%; palm oil 0.5%; pH 6.2-6.4. The vegetative inoculum of 48 hrs. shaken cultures was used at the rate of 1 per cent (v/v) to inoculate 100 ml fermentation medium. The inoculated medium contained 8 $\mu\text{g}/\text{ml}$ of chloride.

Results

In the first part of the experiments the effect of chloride ion concentration on CTC biosynthesis was studied. It was found that adding at different times an excess of chloride (in the form of sodium chloride) to cultures growing on chloride deficient media, a significant increase in tetracycline (TC) concentration occurred, even after the chloride had been added (Table I).

Table I
Effect of NaCl on the biosynthesis of tetracycline***

Total antibiotic production		Time of NaCl addition	Total antibiotic production after NaCl additions, until the 96hr, $\mu\text{g/ml}$	TC production after NaCl additions	
Age	$\mu\text{g/ml}$			$\mu\text{g/ml}$	per cent
—	—	0h	1550	30	2
24h	240	24h	1310	195	15
42h	720	42h	830	240	29
66h	1200	66h	350	175	50
74h	1320	74h	230	140	60
96h	1550	—	—	1457	93

* NaCl was applied at the rate of 0.4 per cent.

** Beside CTC exclusively TC was formed in these experiments.

It is seen from *Table I* that the amount of TC was increasing in every case, even after the chloride had been added. Within the total amount of tetracyclines formed following the addition of chloride the relative quantity of TC increased in proportion to the time of chloride addition. Furthermore, if an excess of chloride had been introduced in the active phase of TC synthesis e. g. at the 42nd hr and samples were taken afterwards from time to time for the assay of the CTC/TC ratio, the quotients obtained at different periods showed an increasing tendency (*Table II*).

Table II
Influence of NaCl on the CTC/TC quotient*

Total antibiotic production		Total antibiotic production after NaCl addition		Antibiotic composition after NaCl addition		CTC/TC quotient (q)
Age	Conc. $\mu\text{g/ml}$	Time of assay	Conc. $\mu\text{g/ml}$	CTC $\mu\text{g/ml}$	TC $\mu\text{g/ml}$	
42h	410	—	—	—	—	—
48h	625	6h	215	87	128	0.67
54h	830	12h	420	210	210	1.00
66h	1040	24h	630	355	275	1.29
78h	1165	36h	755	840	275	1.75
90h	1345	48h	935	660	275	2.40

* NaCl was introduced at the 42nd hr, at the rate of 0.4 per cent.

If following the addition of sodium chloride there were no more formation of TC, the q values ought to be theoretically infinite, but practically about 19–20.

Experiments were carried out to test the role of TC, in which pure TC (200–1000 $\mu\text{g/ml}$) was added to cultures growing on chloride rich media and producing only CTC, but the added TC could be detected at the end of the fermentation and no increase in CTC concentration at the expense of TC was observed. Within certain concentrations limits strain B-28 was able completely to utilize the chloride of the medium for the synthesis of CTC. In this case the amount of synthesized CTC was proportional to the chloride concentration of the medium. On this basis a quantitative method was worked out for the microbiological determination of small amounts of chloride [7]. Regarding its intensive chloride utilization strain B-28 belongs to the first class of DOERSCHUK *et al.* [6].

Next, various halide homologues, were studied for their effect on synthesis of tetracyclines in the presence of a given chloride concentration. The data are summarized in *Table III*.

Table III
*Effect of halide homologues on the synthesis of CTC**

Concentration of halides gEq	Percentage of TC		
	Fluoride (NaF)	Bromide (KBr)	Iodide (KI)
0.04	—	75	48
0.02	60	67	39
0.01	42	58	35
0.005	33	45	33
0.0025	30	32	31
0	25	25	25

* Experiments were carried out in the presence of 2 mEq NaCl.

The results given in *Table III* show a fair agreement with those reported earlier [1–4], only the relations of the CTC/TC ratios to the halide concentrations show some quantitative difference.

Starting from the earlier observation of SEKIZAWA [5], some physiologically active compounds, also known as copper reagents, were tested for their effect on CTC synthesis (*Table IV*).

The data of *Table IV* show that thioglycolic acid and cyanide itself are ineffective, but administered together with bromide they enhance the effect of the latter. *Cupferron* (NH_4 salt of phenyl-nitrosohydroxylamine) itself is slightly inhibitory and this effect is increased by bromide. Results similar to those of SEKIZAWA [2–4], and POPOVA [5] were obtained with 2-mercapto-benzothiazole and thiocyanate. In agreement with SEKIZAWA it was found that the inhibitory effect of 2-mercapto-benzothiazole on CTC synthesis can

Table IV

Effect of various compounds on the synthesis of CTC

Compound tested	Percentage of CTC formed	
	without bromide	in the presence 0.02 M bromid
Control	75	33
Thioglycolic acid 5 µg/ml	75	23
Thioglycolic acid 100 µg/ml	75	15
NaCN 10 ⁻⁴ M	75	25
NaCN 10 ⁻⁵ M	75	33
Benzaldoxime 25 µg/ml	68	26
Cupferron 100 µg/ml	75	24
Cupferron 200 µg/ml	68	18
2-mercapto-benzothiazole 10 µg/ml ...	60	7
2-mercapto-benzothiazole 30 µg/ml ...	50	3
NH ₄ SCN 500 µg/ml	50	22
NH ₄ SCN 1000 µg/ml	55	17

be partly reversed by the addition of Cu⁺⁺ ions. Introducing copper and 2-mercapto-benzothiazole in the molar ratio of 1 : 2, a 12 per cent increase of CTC production occurred in the copper containing flasks as compared with the control with 2-mercapto-benzothiazole alone. Working with the CTC producing strain, *Streptomyces sayamaensis* and using copper and 2-mercapto-benzothiazole in the molar ratio of 1 : 1.5 SEKIZAWA [5] found a hardly estimable difference of 4 per cent. This might have been due to the fact that in his experiments the percentage of TC was too high, 95 per cent or more, and accuracy of the known analytical methods is considerably reduced at such limits.

Table V

The influence of copper (II) on CTC synthesis in the presence of 2-mercapto-benzothiazole***

Substrate	Total antibiotic µg/ml	CTC		TC	
		µg/ml	%	µg/ml	%
Control	1480	1120	75.6	360	24.4
2-mercapto-benzothiazole 10 µg/ml	1210	725	60.0	485	40.0
2-mercapto-benzothiazole 10 µg/ml + Cu ⁺⁺ 2 µg/ml	1150	830	72.2	320	27.8

* Copper was applied in the form of CuSO₄.

** Each flask contained 2.5 mM NaCl.

Discussion

When the foregoing data of the present experiments, concerned with the effect of chloride on CTC synthesis, are considered it is evident that incorporation of the chlorine atom does not take place on the complete TC molecule. Considering that TC was synthesized for a relatively long period even after an excess of chloride had been introduced into cultures growing on chloride deficient media, as well as that the CTC/TC quotient was very low after chloride addition, it seems probable that chlorination occurs at a relatively early stage of TC biosynthesis which is then followed by more steps. From this it follows that those intermediate compounds of the biosynthetic chain which had passed the critical chlorination phase before the chloride was added, cannot any more serve as substrates for chlorination, and synthesize TC only. This conclusion, however, cannot be brought into harmony with the recent view of ABRAHAM and NEWTON [8] that chlorination would take place at a late stage of CTC synthesis.

Our experimental findings with halide homologues support the earlier statements that incorporation of chlorine into CTC is inhibited competitively by the other halides in the following order: $\text{Br}^- > \text{F}^- > \text{J}^-$.

Our results with metal complexing compounds and with copper ions supported the hypothesis of SEKIZAWA, that copper participates in the chlorination process. However, neither SEKIZAWA's results nor our own experimental findings have furnished final evidence of the role of copper being connected with the participation of a copper-enzyme. This question remains to be elucidated.

Examination of the chlorination process from the genetic point of view yielded the finding that specially applied mutagenic interactions combined with carefully selected biochemical treatments can under certain conditions induce a mutant with considerably reduced chlorinating activity, insignificant in comparison with that of the parent strain. This observation allows the conclusion that the chlorination mechanism in the biosynthesis of CTC can be genetically influenced.

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Address of the authors: GYULA KOLLÁR, MIKLÓS JÁRAI,
Biochemical and Microbiological Laboratories, Department of Antibiotics,
"Chinoïn" Chemical and Pharmaceutical Works, Budapest IV. Tó u. 1/5.

THE DETECTION OF UNKNOWN ENTERIC PATHOGENS BY CONJUNCTIVAL INFECTION OF GUINEA PIGS

By

B. RÉDEY and F. CSIZMAZIA

Public Health Laboratory, Veszprém

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Summary. (i) Two different types of *E. coli* have been observed to cause artificial purulent keratoconjunctivitis in guinea pigs. The ability of causing keratoconjunctivitis not being restricted to the genus *Shigella*, systematically different organisms may on the basis of the eye-test be included in one pathogenetic unit.

(ii) The conjunctival infection of guinea pigs seems to offer means for isolating *Shigellae* at present unknown or undetectable by the usual methods. Furthermore, it appears to be a selective method for tracing virulent bacteria of other groups, whether or not they are known at present to be capable of causing dysenteric symptoms. The positivity of the reaction should be regarded as a sign of pathogenicity and virulence of the microorganism tested.

In 1924, French authors [1] showed that the inoculation of certain *Shigella* strains into the eye of guinea pigs which had been pre-treated with bile caused purulent keratoconjunctivitis. In 1943-44 BINGEL [2] found that in guinea pigs purulent cystitis followed the introduction of freshly isolated *Shigella* strains. This method proved suitable for differentiating between virulent and avirulent *Shigella* strains, and theoretically it would have been useful in the research of the problem of dysentery. Because of its difficult control, however, it was not applied as a routine test.

In 1954, SERÉNY [3] claimed to have shown independently from the French authors that in the guinea pig *Shigella* strains gave rise to purulent keratoconjunctivitis. In contrast to the French workers he carried out the experiments on untreated eyes, and found that from among the numerous enteric bacteria tested, only *Shigellae* gave a positive reaction. The eye-test is easy to carry out and the results are well definable. It has the advantage of BINGEL's reaction, being suitable for differentiating between freshly isolated virulent and avirulent laboratory cultures. Recognizing the importance of the test, SERÉNY called attention to its wide range of applicability. After his first paper, other authors have also proven the value of the eye-test in experimental and routine work [4].

In the present paper, attempts at the extended use of the method are described. (i) In contrast to earlier views, the reaction was not employed to determine whether an isolated strain belonged to the *Shigella* group. It was used for showing the ability of causing dysenteric disease of bacteria belonging to any of the enteric groups. (ii) The eye-test was employed as a selective

diagnostic procedure for tracing new *Shigella* strains or *Shigella* undetectable in faecal samples by the usual methods. The reaction was used also to detect known or unknown bacteria of other enteric groups which produced dysenteric enterocolitis.*

Materials and methods

Two kinds of methods were applied as follows.

a) Provided that at the routine examination of a faecal sample no characteristic colonies had appeared on desoxycholate citrate (DC) agar plates, the growth consisting of various enteric bacteria was transferred onto a blood agar plate. After 14 to 15 hours of incubation several loopful of the subculture was inoculated into the conjunctival sack of guinea pigs.

b) Alternatively, the faecal specimen was directly streaked on blood agar. The growth was then inoculated into the eye of a guinea pig. Before transfers or inoculation into the eyes the growth on the surface of the corresponding plates was well mixed by a loop. Administration of the infective material was performed as described by SERÉNY. On the next day, the discharge from the eyes was streaked on DC eosin-methylene-blue or other media used for enteric bacteria. In positive cases the plates inoculated this way contained the causative agent mostly in pure or nearly pure culture and by this time the eyes usually showed signs of keratoconjunctivitis. In order to prove that the agent isolated was the particular one actually responsible for the eye-reaction, the test was repeated with the pure culture. From the infected eyes the causative organism could be isolated over a considerable length of time.

Since the infection can spread from one animal to the other, during the experiments the guinea-pigs were strictly isolated. The infection was spread mainly with flying hairs, rarely with cough and sneezing after an eventual infection of the nasopharyngeal tract. *Salmonellae* also multiply in the eye of the guinea pig; in this case, only conjunctivitis develops. The reaction caused by *Salmonella* may perhaps offer another possibility for the method's application. (Considering the risk of infection, e. g. with *S. typhi*, caution is recommended.)

The biochemical examinations were performed according to KAUFFMANN [5]. The serological tests were carried out as described by EDWARDS and EWING [6].

Results

1. Tracing *Shigellae* producing atypical colonies or overgrown by other bacteria.

a) According to epidemiological examinations, a typical outbreak of dysentery was suspected in a village. The routine examination of the faecal specimens sent to this laboratory revealed no pathogenic bacteria. Cultures from three subsequent faecal samples were immediately put up, but despite careful studies no pathogenic bacteria could be isolated. The samples were then streaked on blood agar and the growth was transferred into the eyes of guinea pigs. As one of the samples caused an inflammatory reaction, the discharges were seeded on media on which finally pure cultures of typical *Sh. flexneri* strains were isolated. The revision of the original, at that time 4 days old, DC plates disclosed some colonies on one of them. According to

* The term "dysenteric diseases" means a pathological condition in which — like in typical dysentery — the pathological and bacteriological changes are localized chiefly in the colon.

the biochemical and serological examination, these corresponded to *Shigellae*. These colonies had been nearly invisible at the usual 24 and 48 hour readings. Because of their minuteness they did not resemble *Shigella* colonies even on the fourth day. This atypical appearance of pathogens is familiar to the bacteriologists [6, 7].

b) In *Pápa*, a town belonging to the district of our Department, an outbreak due to *Sh. sonnei* occurred. The faecal specimens negative by the usual methods were streaked on separate blood agar plates. The growth from 10 plates (cultured from the faeces of 10 different patients) were mixed and the mixture was inoculated repeatedly into eyes. Positive reactions were demonstrated in 2 out of 4 such experiments, one of them giving *Sh. sonnei*, the other *Sh. flexneri*. The latter organism occurred rarely in the town at that time. By inoculating instead of the blood agar cultures the growth from 24 hours DC plates the same result was obtained.

2. Tracing unknown pathogens belonging to the *E. coli* group.

From a village of our district (*Öskü*) a faecal specimen was received. This had been taken from a 8 year old girl on the first day of the onset of a disease diagnosed as dysentery. The patient complained of vomiting and frequent diarrhoea; she had a temperature of 37.8° C. The specimen was streaked as usual on DC, bismuth sulfite and eosin-methylene blue media. On the plates among other enteric bacteria typical *E. coli* colonies were observed. No *Shigellae* were found. The growth from the DC plate was collected and densely inoculated onto blood agar. The growth from the latter was transferred into the eyes of a guinea pig, where it caused typical keratoconjunctivitis. From the discharge *E. coli* was cultivated. With a culture made from the colonies of the latter a successful conjunctival infection was brought about. Accordingly, this *E. coli* strain was supposed to be pathogenic. Control examination of the healthy contacts showed that the patient's 6 year old sister was harbouring the same bacterium. When the sample had been taken, this child had shown no symptoms of illness. Some hours later, however, she developed mild diarrhoea, similar to what her sister was suffering from. The *E. coli* strain isolated from the latter child also gave a positive eye-reaction. In the family of these two children a symptomless carrier was in addition detected. All of these *E. coli* strains gave rise in the eyes of guinea pigs to typical keratoconjunctivitis which, however, was milder and recovered more rapidly than the infections caused by *Shigellae*.

In searching for the organism in the kindergarten of the same village, 4 out of 45 specimens were found positive. According to the nurse, only one of these children had diarrhoea. The same bacterium was isolated from the water of a well, which the two children with enteritis and their family had been consuming. From the brook traversing the village the organism was

also isolated. Both of the latter bacteria gave positive eye-test. Thus the organism was widely present in the village at the time of the investigations. Attempts were made to isolate it from materials obtained from other places. *E. coli* strains having a similar colonial appearance and agglutinating in a serum prepared with the original culture were selected. In some cases strains were met with which, according to the slide agglutination, were related to or were identical with the pathogenic culture. These strains had been isolated from healthy individuals and they always gave negative eye-reactions. Their antigenic structure has not been analysed.

The pathogenic strains were typical *E. coli* cultures. On DC and other media for enteric bacteria they formed colonies similar to those of other *E. coli* strains. Biochemically they also belonged to the Escherichia group. The reactions given by them were as follows. Lactose, glucose, galactose, mannitol, sorbitol, trehalose, arabinose, raffinose, rhamnose and d-xylose were fermented within one day with acid and gas production. Dulcitol, inositol, inulin, salicin and adonitol were not attacked. In sucrose a weak acid reaction without gas formation ensued in two weeks. Urease was negative, H_2S negative, indole positive, Voges—Proskauer negative, methyl red positive. No growth was observed in KOSER's citrate and in KCN medium. Nitrates were reduced. The forms were non-motile. Pathogenic *E. coli* strains 018, 026, 044, 055, 086, 011, 0112ac, 0119, 0124, 0125, 0126, 0127, 0128 and 0135 showed no agglutination in a serum prepared with the living culture of the original strain. Neither the living nor the boiled culture of the strain reacted with polyvalent Shigella sera (*Sh. dysenteriae* 1—7, *Sh. flexneri*, *Sh. boydii* 1—7, *Sh. sonnei* I—II)

The pathogenicity of the organism was tested in human volunteers. On the first occasion 2×10^9 living cells were administered by mouth. After 24 hours colic and watery stools developed. The temperature was $38^\circ C$. The stools contained the organism in almost pure culture (1×10^7 cells per gram of faeces). Then the discomfort diminished and the mass excretion of the pathogenic strain ceased. After four days the same individual was given 5×10^9 organisms. After 24 hours severe enterocolitis developed with pyrexia up to $39^\circ C$. Bloody diarrhoeal stools were excreted eight times a day. Of the administered *E. coli* strain the faeces contained 2×10^8 cells per gram. The temperature gradually decreased and on the fourth day it became normal. Recovery was relatively rapid without any rational treatment. The organism was not isolated after the sixth day. Blood cultures taken several times during the incubation period and the illness remained sterile. The number of leucocytes was 20 000 per μl of blood at the peak of pyrexia.

The pathogenicity of the organism was thus verified. It will be considered only after performing the adequate serological analyses whether the strain represents a known *E. coli* serotype so far not associated with enterocolitis, or a new serotype with pathogenic properties.

3. *Confirming the ability to cause dysenteric symptoms of freshly isolated strains.*

a) From the village Dudar faeces were received from a child showing the clinical signs of dysentery. On bacteriological examination no Shigellae were found. The typical *E. coli* strain isolated from the faeces caused inflammation in the eyes of a guinea-pig. Serological examination revealed the identity of this organism with the *E. coli* strains isolated in Öskü. The disease caused was mild, similarly to that given rise by the Öskü strains.

b) In the summer of 1958, due to the pollution of the water-works a mass outbreak of enteritis occurred in Veszprém [8]. From the faeces of the patients an organism antigenically related to Shigellae was isolated. Later it was identified as belonging to *E. coli* 0 group 124. Since the pathological changes due to *E. coli* 0124 in the intestinal tract of man are similar to those produced by Shigellae, some strains were tried in conjunctival infection. Typical keratoconjunctivitis developed, confirming the responsibility of the organism for the outbreak.*

The *E. coli* 0124 strain Sz. 740 examined by KÉTYI *et al.* [9], which had caused some laboratory infections, was also found capable of giving rise to keratoconjunctivitis.

Discussion

In the present examinations the eye-test has been used for solving problems different from the original purpose of the method. Our experience does not yet permit to recommend the technique ensuring the best results. The method might be made more effective by altering some of its phases. *E.g.* media may be found which before the conjunctival infection promote the multiplication of the pathogenic agent but not of the other enteric bacteria. The material to be inoculated may perhaps be mixed with some special bacteria which would increase the probability of infection. In model experiments it should be attempted to make the eye of the guinea-pig more sensitive to infection, eventually by pre-treatment with bile, other bacteria, substances causing inflammation, etc.

Experiment 1b shows the efficiency and sensitivity of the method. In this case only out of the 10 faecal samples probably contained Shigellae. On cultivation the pathogenic agent must have formed few colonies only and these were presumably overgrown by other bacteria. Yet, on inoculating the Shigella-containing specimen diluted with the other nine negative samples, a positive eye-reaction resulted. (It should be noted that in this experiment a considerable amount of material was used repeatedly for the conjunctival infection). It is

* From other reasons some of these *E. coli* 0124 strains were sent to DR. B. SERÉNY, State Institute of Hygiene, Budapest, who independently from us also showed their pathogenicity to the guinea pig's eye.

clear that while the examination of separate samples requires one guinea pig each, the inoculation of pooled material has made possible to isolate the pathogen from the faecal flora of as many as ten individuals. The easy technique of the method might contribute to the elucidation of some important problems concerning natural infection. Such investigations are in progress in this laboratory.

The method presented seems to be suitable for tracing *Shigellae* in cases when these bacteria would have otherwise been missed. *Shigella* colonies on the usual selective media may escape attention owing to the atypical growth of some strains. On the other hand, when very small number of *Shigellae* are present in the faeces, they may grow concealed among the colonies of other enteric bacteria. In these instances the pathogen will still be selected by the highly susceptible eye of the guinea pig. The result of Experiment 1a may be interpreted so that in the faecal sample a pathogenic *Shigella* strain forming dwarf colonies was present. (Organisms growing in dwarf colonies are regarded non-pathogenic by WAALER [10]. However, pathogenic *S. typhi* strains forming dwarf colonies are known to occur.) Alternatively, typical *Shigella* colonies may be overgrown by other enteric bacteria. Although neither of the two possibilities have been proved experimentally, in Experiment 1a the former, in Experiment 1b the latter possibility had probably been the case. At all event, the test with its present technique seems to be more sensitive than any of the methods so far known.

The guinea pig eye-test was regarded by some authors as specific for *Shigellae* and on the basis of a positive reaction they classified *Shigella*-like strains into the genus *Shigella*. From the present studies, however, it is clear that regardless to whether or not the organism belongs to the genus *Shigella*, the positivity of the eye-test runs parallel with the strain's ability of causing enterocolitis in man. The severe lesions and the incidence of disease caused by *Shigellae* show that these organisms are the most important causative agents both of the artificial keratoconjunctivitis of guinea pigs and the enterocolitis of man. The biological reactions of the human colon and those of the guinea pig's eye are certainly not identical; nevertheless, with the aid of the simple eye-reaction the organisms capable of causing dysenteric enterocolitis may be included in one pathogenetic group, — irrespective of their systemic position.

The pathogenic role of certain freshly isolated strains may occasionally be suspected on the basis of clinical, epidemiological, biochemical or serological reasons. Identifying and proving the pathogenicity of such strains is, however, time consuming and often difficult to perform (lengthiness of biochemical reactions, lack of special agglutinating sera, etc.). These strains therefore are often unidentified. On the other hand, a positive result yielded by the rapid eye-test will indicate the pathogenicity of the agent. In this case the identi-

fication of the strain and the examination of its pathogenicity for humans should by all means be carried out. A negative reaction does not of course exclude the pathogenic role of any organism. The eye-test has the advantage that it calls attention to the virulent strains belonging to groups the members of which are generally facultative pathogens (*E. coli*, etc.). On the basis of the usual methods of identification, these would likely to pass as non-pathogenic bacteria.

To draw final conclusions as to the parallelism between the pathogenicity of bacteria to the guinea pig's eye and to human beings experiments on volunteers must be made. SERÉNY's investigations have made probable the association between the positive eye test and the virulence of Shigellae. Our experiments [11] on human volunteers have shown that the positivity of the eye-test is in a much closer connection with the pathogenicity of Shigellae to man than of any other test so far known. In these experiments seven laboratory stock cultures giving negative reactions (*Sh. flexneri* and *Sh. sonnei* phase 2) were administered by mouth. Considerable quantities of these bacteria failed to cause clinical symptoms. The association between the eye-test's outcome and the pathogenicity to man was shown also by the following observation. Generally, all *Sh. sonnei* phase 1 cultures are believed to be virulent. The loss of virulence is supposed to be connected with a dissociation from phase 1 to phase 2 or to the *R* form. No clinical symptoms developed in man after the oral administration of high quantities of *Sh. sonnei* phase 1 bacteria which gave a negative eye-test. The same strain showed a high virulence to the mouse; this animal was killed by one cell injected with mucin intraperitoneally. Strains pathogenic to man could much better be differentiated by the eye-test than morphologically, biochemically, serologically or by the usual methods of animal inoculation [11]. Some special morphological and biochemical procedures might perhaps reveal differences between the two kinds of *Sh. sonnei* phase 1 cultures (virulent and avirulent by the eye-test). The above volunteer experiments have shown the negative side of the problem *i. e.* that strains giving a negative reaction were non-virulent to man. However, in the present paper, it has been demonstrated that the reaction can adequately be used not only for showing differences in virulence, but also for examining the pathogenicity and virulence of strains to man.

Freshly isolated Shigellae from man were all virulent by the eye-test. Recently, however, NOSKOV [13] isolated from symptomless carriers some strains which gave a negative eye-test. The virulence or the avirulence to man of the latter strains may be shown by human volunteer experiments. The proportion of the strains virulent to the guinea pig's eye and to man among freshly isolated *E. coli* 0124 and *Coli* "Öskü" cultures is being studied.

The positive eye-test given by *E. coli* 0124 isolated from the water-borne outbreak in *Veszprém* confirmed the causative role of this organism. The antigenic structure of *E. coli* 0124 is identical with *Sh. dysenteriae* 3 and

its cultural properties are also similar to those of the Shigellae. The positive test given by these strains would not *per se* suffice for concluding that pathogenic bacteria other than Shigellae are also capable of giving rise to keratoconjunctivitis. A biochemically and culturally typical *E. coli* strain giving a positive eye-test was, however, isolated during the present investigations. In spite of its having been non-motile, it was not related to Shigellae. Organisms giving a positive eye-test undoubtedly occur in the Escherichia group. *E. coli* strains 018, 044, 086, 0112 a. c., 0119, 0126, 0127, 0128 and 0135, which are regarded pathogenic, uniformly gave negative reactions. It should, however, be considered that even among the Shigellae it is the freshly isolated strains which cause keratoconjunctivitis, and as the above strains were old laboratory cultures, the examinations should be repeated with freshly isolated strains. Considering the various kinds of relationship among the enteric bacteria, strains giving a positive eye-test may occur also in other Enterobacteriaceae groups or in entirely different groups of bacteria such as *Listeria monocytogenes* [12], etc.

It would be interesting to collect organisms giving a positive eye-test. The comparative examination of these bacteria belonging probably to various enteric groups, may reveal the common property responsible for the effect exerted on the eye of the guinea pig and the human colon. Provided that the hypothesis advanced in this study will be confirmed by extended investigations, the method will certainly be applicable in practical use. Its high selectivity may facilitate the discovery of new organisms inducing gastroenteritis and thus to detect the causative agents of epidemics, water- and food-borne outbreaks and some other infections of unknown aetiology. By adapting it to some different kind of animal, the test might prove be useful also in veterinary practice.

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Address of the authors: BARNABÁS RÉDEY, FERENC CSIZMAZIA,
Public Health Laboratory, Veszprém, Hungary

PRODUCTION OF INDUCTIVE AMYLASE BY *PENICILLIUM CHRYSOGENUM*

By

I. HORVÁTH, A. SZENTIRMAI, S. BAJUSZ and EVA PARRAGH

Research Institute of the Pharmaceutical Industry, Budapest

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Summary. (i) Production of amylase by washed mycelia of *Penicillium chrysogenum* is of an inductive character.

(ii) The presence of starch is required only for induction; enzyme production proceeds also in the absence of starch.

(iii) Glucose inhibits the inductive phase of enzyme production, but exerts no influence on the production of enzyme.

(iv) Mycelia grown on starch produce little amylase. After washing, such mycelia can again be induced with starch.

(v) Inductive production is inhibited by 2,4-dinitrophenol, sodium azide, potassium cyanide, furthermore hydroxylamine, phenylhydrazine, and amino acid esters containing a reactive amino-group.

Penicillium chrysogenum and other fungi have for a long time been known to produce amylase. This industrially important question has been dealt with in numerous reports, with special attention to investigations into the production of amylase by *Aspergillus oryzae*. Experiments on different culture media indicate that the production of this enzyme is of inductive character [1-7], as has been proved also by means of washed mycelium [8].

Production of amylase by *Penicillium chrysogenum* has already been described [9]; the present report deals with the inductive amylase production by washed mycelia. Experimental conditions were so chosen as to prove the inductive production of that enzyme. Production of inductive amylase by washed mycelium grown on starch has been studied in detail. Some of the results have already been published [10, 11].

Materials and methods

Penicillium chrysogenum strain Wis. 49-133 was used. Spore production was carried out on potato infusion agar. The mycelium was grown on a rotary shaker (300 r. p. m., 2 cm radius) at 24°C in 200 ml of medium in 500 ml Erlenmeyer flasks. One ml spore suspension with a concentration of 10^4 per ml was used for inoculating an inoculum medium of the following percentual composition: glucose, 2; sodium nitrate, 0.3; potassium dihydrophosphate, 0.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; potassium chloride, 0.05; pH, prior to sterilization, 6.5; 0.5 per cent CaCO_3 was added before inoculation. To produce mycelia, 10 ml of a 72-hour vegetative culture were inoculated in 200 ml of the above medium. The carbohydrate source varied with each experiment, but the concentration was always 2 per cent.

The obtained mycelia were filtered through cloth, washed in distilled water of sixfold volume, and suspended in distilled water to make up the dry matter content to 1.0 to 1.3 g

per 100 ml. Enzyme production took place on a rotary shaker in 500 ml Erlenmeyer flasks in 100 ml volume at 24°C. In most of the experiments the flasks contained 25 ml a 0.4 per cent starch solution and 0.4 per cent potassium dihydrophosphate, with the pH adjusted by potassium hydroxide to 6.5 before sterilization. 50 ml of mycelium suspension were added, as well as 25 ml distilled water (the added materials were always dissolved in this volume). (In the following the final concentrations will be given.) The experiments were performed under aseptic conditions and tested several times for sterility.

Samples taken at appropriate intervals were filtered through paper. Amylase activity was determined in the filtrate at 28°C by the method of SMITH and ROE [12].

The mycelial dry-material content was determined by drying at 105°C after filtration and washing with 10 per cent acetic acid. The amount of mycelium is indicated below the figures. The quantity of enzyme obtained has not been computed for the mycelial dry material, since the amount of the produced enzyme does not depend on the latter within the amount of the dry substance.

The intracellular amino acid content of the mycelia was determined by various extraction methods. These methods included boiling for thirty minutes in distilled water and boiling for the same period in 75 per cent alcohol. Determination of amino acids in the extracts was carried out by the method of BOISSONAS [13], and was calculated for glycine.

The amino acid esters were synthesized by the usual methods [14–19].

Experimental

Penicillium chrysogenum grown on synthetic media containing various sources of energy (glucose, sorbitol, glycerol, lactose) do not produce amylase during growth. When two-day-old mycelia are washed and suspended in starch solution containing phosphate buffer, enzyme production begins after four hours' incubation, reaching 150 to 250 U per 100 ml in another four to

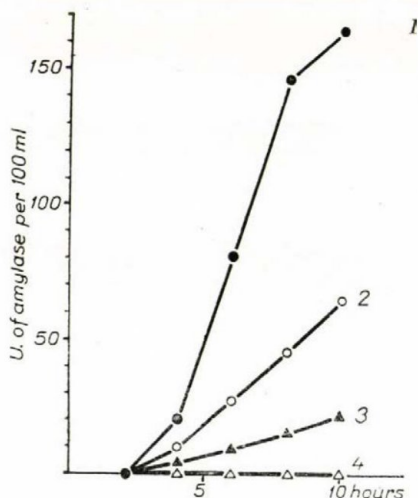


Fig. 1. Effect of starch concentration on induced synthesis of amylase. The incubation solution contained 0.1 per cent KH_2PO_4 (pH 6.5 with KOH), 0.62 g per 100 ml washed mycelium (drymatter), and various amounts of soluble starch.

- Signs: 1: 0.1 per cent starch
 2: 0.05 per cent "
 3: 0.01 per cent "
 4: no starch

six hours; this value is maintained over a minimum of twenty hours. Neither at the beginning of the experiments nor at their end was amylase demonstrated in mycelia exposed by grinding with glass powder. The production of enzyme depends on the presence of starch; with starch concentrations between 0.1 and 2 per cent, equal amounts of enzyme were obtained. In the case of lower starch concentrations production was found to diminish (*Fig. 1.*).

Repeated washings following induction have shown that the presence of starch is needed only for induction; enzyme production itself proceeds also

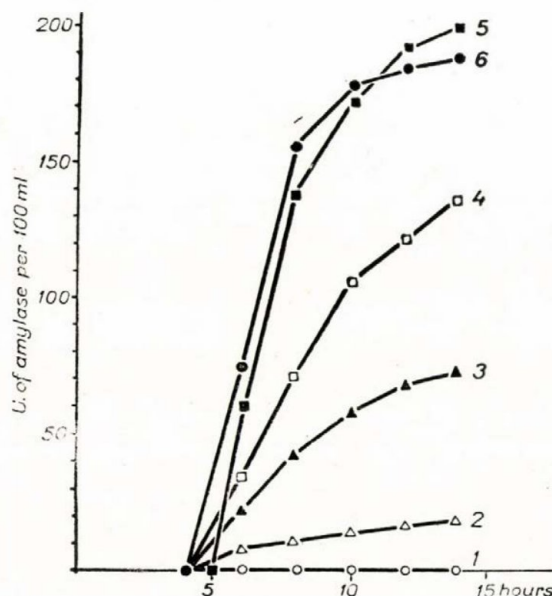


Fig. 2. Effect of second washing after incubation on induced synthesis of amylase

The mycelium was induced with a solution containing 0.1 per cent KH_2PO_4 (pH 6.5 with KOH) and 0.1 per cent soluble starch. Concentration of mycelium (dry-matter), 0.55 g per 100 ml. Induced mycelia were washed at various points of time and suspended in a solution containing 0.1 per cent KH_2PO_4 (pH 6.5 with KOH).

- Signs: 1: second washing at 0 hour
 2: " " " 2 hours
 3: " " " 3 hours
 4: " " " 4 hours
 5: " " " 5 hours
 6: unwashed control

without starch. The results summarized in *Fig. 2* demonstrate that there was no enzyme production in phosphate buffer lacking starch when the mycelium had been washed between 0 and 2 hours; reduced enzyme production occurred after washing between 2 and 4 hours, whereas after washing in the 5th hour enzyme production was unchanged.

The inductive phase of enzyme production was very sensitive to the presence of glucose. Production of amylase was inhibited by the presence of

0.1 per cent glucose at the time of induction. However, the process of enzyme production was not sensitive. In the presence of 2 per cent glucose the rate of enzyme production was unaltered. If the induced mycelia were washed at 4 to 5 hours and suspended in a starchfree medium with glucose, or glucose was added simultaneously to the culture, enzyme production was unchanged. *Fig. 3* illustrates the effect of 0.1 per cent glucose added at various intervals: inhibition of enzyme production was complete when glucose had been added between 0 and 2 hours, and only partial after addition between 2 and 4 hours.

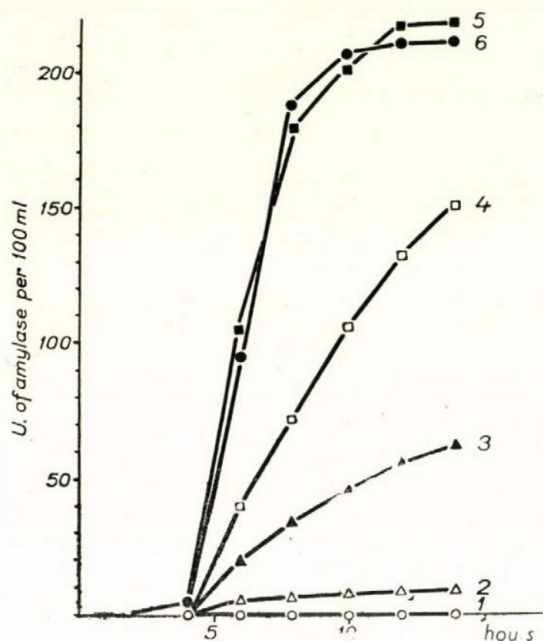


Fig. 3. Effect on induced amylase synthesis of glucose added at various points of time. The mycelium was induced by a solution containing 0.1 per cent KH_2PO_4 (pH 6.5 with KOH) and 0.1 per cent soluble starch. Mycelium concentration (dry-matter) 0.56 g per 100 ml. Glucose added at various points of time amounted to 0.1 per cent

Signs: 1: addition of glucose at 0 hour 4: " " " " 4 hours
 2: " " " " 2 hours 5: " " " " 5 hours
 3: " " " " 3 hours 6: control

Glucose added after 4 hours brought about no inhibition. 2 per cent glucose had the same effect.

If starch was added to the washed and phosphate buffered mycelium in the first six hours of incubation, enzyme production was similar but between 2 and 4 hours the rate of synthesis was more rapid. Markedly decreased production may be observed when starch is added in the eighth hour or later (*Fig. 4*).

During the experiments the pH of the medium varied between 6.2 and 6.6; had at the start the pH been adjusted to a different value it returned

to the above level in the first two or three hours of incubation. Thus there was no possibility of investigating the correlation between enzyme production and pH.

The influence of the age of mycelia on enzyme production was also studied. The experiments summarized in *Fig. 5* show that enzyme production of inductive character was similar, but following incubation young, 28-hour-old aged 96-hour-old and partly autolyzed mycelia yielded very little, 20 to 40 U. per 100 ml, enzyme.

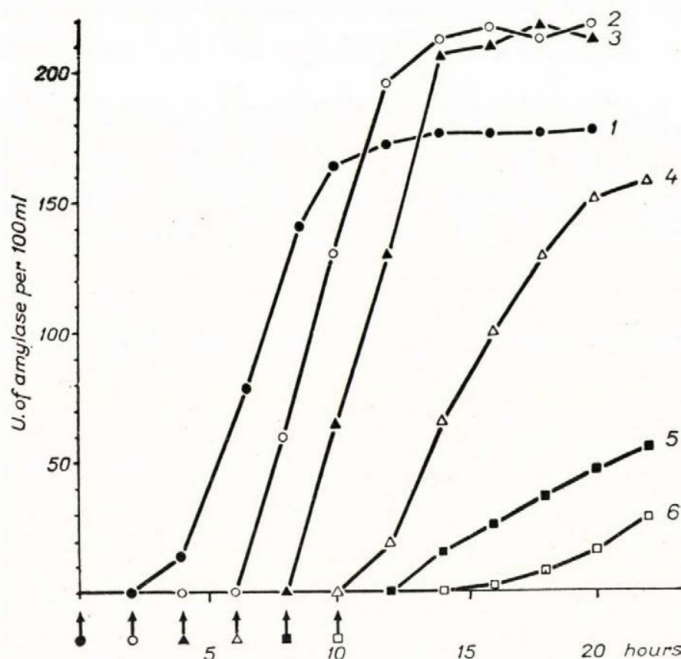


Fig. 4. Effect of "starvation" prior to induction on induced synthesis of amylase
To washed mycelium (dry-matter content 0.60 g per 100 ml) suspended in a solution containing 0.1 per cent KH_2PO_4 (pH 6.5 with KOH), 0.1 per cent soluble starch was added at various points of time

Signs: 1: starch added at 0 hour
2: " " " 2 hours
3: " " " 4 hours
4: " " " 6 hours
5: " " " 8 hours
6: " " " 10 hours

(Time of addition indicated by arrow)

Enzyme production was not increased by the addition to the incubation fluid of substances containing organic or anorganic nitrogen or fibrin hydrolysate. These materials failed to influence enzyme production even when the yield was small owing to the mycelium being young or old, or starved after washing.

When the mould was grown in cultures on media containing starch, enzyme production was proceeding at the rate of 0.5 to 2 U. per 100 ml per hour. Mycelium grown on starch, washed, and suspended in a phosphate buffer containing starch produced no appreciable amount of amylase for four hours. Subsequently rapid production occurred at a rate of 30 to 60 U. per 100 ml per hour. In the case of mycelia grown on other carbon sources enzyme synthesis proceeded similarly reaching and maintaining the value of 150 to 250 U. per 100 ml (Fig. 6).

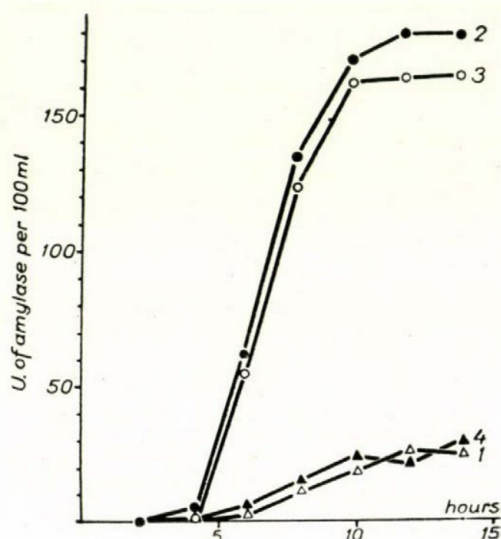


Fig. 5. Effect of the age of mycelium on induced synthesis of amylase
The mycelium was induced with a solution containing 0.1 per cent KH_2PO_4 (pH 6.5 with KOH) and 0.1 per cent soluble starch

Signs:	1:	24-hour-old mycelium	(0.51 g dry-matter per 100 ml)
	2:	48- " "	(0.61 g " " 100 ")
	3:	72- " "	(0.60 g " " 100 ")
	4:	96- " "	(0.59 g " " 100 ")

In view of this behaviour of mycelia grown on media containing starch, studies were carried out in order to determine by means of the iodine colour reaction the disappearance of the starch from the inducing medium.

The results presented in Fig. 6 demonstrate that mycelia grown on starch produce very little enzyme. The starch-iodine colour vanished as early as in the third hour, whereas in the case of mycelium cultivated on glucose, disappearance of the blue colour coincided with the onset of induced enzyme production.

Mycelia of a certain age or starved mycelia yielded reduced amounts of enzyme. The amount of amino acid extracted from the mycelium by boiling

in distilled water or in 75 per cent alcohol was also determined, and the possible existence of a correlation between this value and the production of amylase was investigated. The results are not presented in detail, since variations with the age and condition of the mycelia (15 to 18 mg of amino nitrogen per g of dry mycelium) were strikingly slight, hardly exceeding the experimental error.

The influence of various inhibitory substances on the above mentioned enzyme synthesis was also investigated. The results summarized in *Table I*.

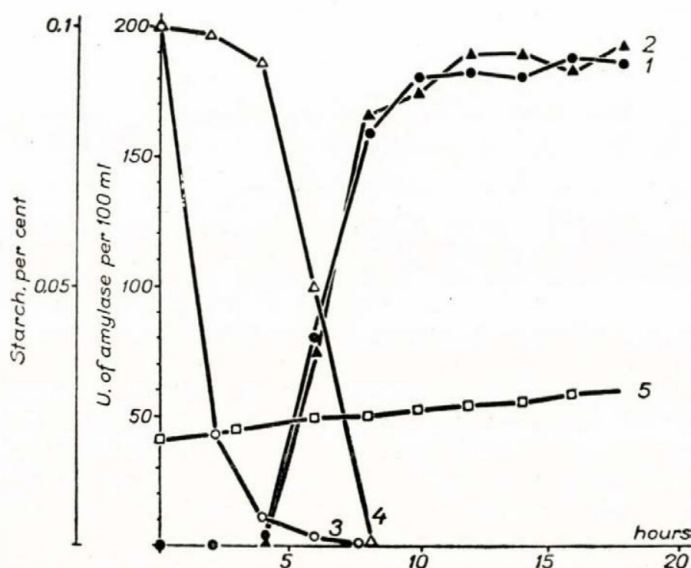


Fig. 6. Synthesis of amylase induced by mycelium grown on medium containing starch. The mycelium was induced with a solution containing 0.1 per cent KH_2PO_4 (pH 6.5 with KOH) and 0.1 per cent soluble starch.

- Signs: 1: amylase production by washed mycelium (0.57 g dry-matter per 100 ml) grown in medium containing starch;
 2: amylase production by washed mycelium (0.60 g dry-matter per 100 ml) grown in medium containing glucose;
 3: disappearance of starch (on the basis of the iodine colour reaction) eliciting induction in the case of mycelium grown in starch medium;
 4: the same in the case of mycelium grown in glucose;
 5: amylase production during the experimental period in the original starch medium (0.38 g dry-matter per 100 ml).

demonstrate that 2,4-dinitrophenol and sodium azide, as well as sodium cyanide acted as inhibitors. Inhibition was brought about also by hydroxylamine, phenyl-hydrazide, furthermore similar concentrations of α -amino acid esters. Inhibition was observed in both the presence and the absence of starch, if enzyme production was determined 4 hours after induction.

Table I
Inhibitory action of various substances on the production of amylase

Name of substance	Concentration	Inhibition per cent
2,4-dinitrophenol	3.10^{-3} M.	95
	1.10^{-3} M.	40
	3.10^{-4} M.	25
Sodium azide	1.10^{-3} M.	100
	2.10^{-4} M.	80
	1.10^{-4} M.	30
Potassium cyanide	1.10^{-3} M.	100
	5.10^{-4} M.	60
	1.10^{-4} M.	30
Phenylhydrazine	2.10^{-3} M.	100
	5.10^{-4} M.	52
	2.10^{-4} M.	18
Hydroxylamine HCl	1.10^{-2} M.	100
	5.10^{-3} M.	90
	1.10^{-3} M.	61
Glycine ethyl ester HCl	1.10^{-2} M.	97
	5.10^{-3} M.	90
	1.10^{-3} M.	61
	5.10^{-4} M.	25
D-phenylalanine methyl ester HCl ...	1.10^{-2} M.	99
	5.10^{-3} M.	96
	1.10^{-3} M.	42
L-isoleucine methyl ester HCl	1.10^{-2} M.	85
	5.10^{-3} M.	55
	1.10^{-3} M.	22
L-tyrosine ethyl ester HCl	1.10^{-2} M.	92
	5.10^{-3} M.	55
	1.10^{-3} M.	23
L-glutaminic acid- γ -ester HCl	1.10^{-2} M.	0
Glycine-amide HCl	1.10^{-2} M.	0
Glycylglycine ethyl ester HCl	1.10^{-2} M.	0
Glycine + alcohol (ethyl)	1.10^{-2} M.	0

Discussion

According to the above results, extracellular amylase production by *Penicillium chrysogenum* is of inductive character. The presence of starch is necessary only for induction, just as in the case of penicillinase production by *Bacillus cereus* [20] or myoinositol dehydrogenase production by *B. lactis* [21] or amylase production by *Aspergillus oryzae* [22]. In the case of *Penicillium chrysogenum*, however, removal of the starch before induced enzyme synthesis had started invariably resulted in a diminished production of enzyme,

while with *Aspergillus oryzae* ten minutes' contact between the inducer and the mycelia sufficed for full induction. This difference indicates that in the case of *Penicillium chrysogenum*, presence of the inducer is indispensable until the development of the enzyme producing system. The development of this system is inhibited by nystatin [24].

TANABE *et al.* [8, 24] found glucose to inhibit amylase production by *Aspergillus oryzae*. If the washed mycelium had been induced with maltose no inhibition occurred. Experiments with *Penicillium chrysogenum* yielded similar results: only the inductive stage of enzyme production was inhibited by glucose, the process of enzyme production itself was not. Our studies revealed the possibility of achieving partial inhibition with glucose added at appropriate points of time between 2 and 4 hours; the same amount resulted of enzyme as that obtained following removal of the inducer by washing.

Most processes of inductive enzyme production are sensitive to glucose, both in the inductive and the productive stage. This phenomenon is ascribed by NEIDHARDT and MAGASANIK [25, 26] to the role of the enzymes in metabolism. No phase of induced penicillinase production is sensitive to glucose [21]. Amylase plays a part in the metabolism of *Penicillium chrysogenum* and is sensitive to glucose according to the above-mentioned generalization, but only in the inductive stage. The process of enzyme production takes place also in the presence of glucose, therefore this stage is like the production of penicillinase [27]. This difference of glucose-sensitive and glucose nonsensitive enzymes mentioned by the above authors may be explained by the fact that amylase is an extracellular enzyme.

As revealed by the present experiments enzyme production is reduced in the case of young mycelia in a stage of vigorous growth, and in old autolyzed material. The latter phenomenon is easy to interpret; the former may presumably be due to deeper metabolic interaction that has not been investigated. Starvation of washed mycelia gives rise to a transient increase of enzyme synthesis; similar observations have been made by ERDŐS and TOMCSÁNYI [28]. When starvation is prolonged, enzyme production is found to decrease.

The inhibitory effects obtained with different materials demonstrate the occurrence of *de novo* protein synthesis.

Induced bacterial synthesis of enzymes is inhibited by sodium azide and 2,4-dinitrophenol [29, 30]. Amylase production by *Aspergillus oryzae* is also inhibited by these substances [31]. A similar inhibition of amylase production occurs by *Penicillium chrysogenum* too. Inhibition was noted also following the use of sodium cyanide, furthermore hydroxylamine phenylhydrazin and amino acid esters. With the amino acid esters, inhibition of enzyme synthesis depends on the α -amino group being free and the adjoining carboxyl group being in ester linkage. This is evidenced by the fact that glycine amide, glycyl glycine ethyl ester, glutamic acid ester are ineffective. The inhibitory

effect of the amino acid esters may be explained as follows. These compounds have a marked reactive amino group which reacts with activated amino acids similarly to hydroxylamine [32, 33] and this is what disturbs protein synthesis. The inhibitory effect of this group of compounds on protein synthesis has been assumed by PERRI *et al.* [34] on the basis of experiments of a different kind.

Our experiments were carried out on mycelia grown in glucose. Repeating them with mycelium grown in starch, instead of the expected prompt production of enzyme the result was the same as before. On the basis of detailed inquiry into this phenomenon the following may be stated. The low enzym production observed upon growth in media containing starch is unchanged after the mycelia have been washed; enzyme production is nevertheless increased by the further addition of starch. This process may run its course in the following manner. The inoculated mycelium in the starch-medium is induced to maximum enzyme production. This is, however, slight at the low concentration of the mycelium. The produced enzyme begins to break down the starch present in the medium, and the development of a system synthesizing additional amylase is inhibited by the released glucose. Apart from these two factors, the concentration of synthesized amylase and the consequent concentration of glucose, the final rate of enzyme synthesis would seem to be somehow influenced by the phase of rapid growth of the mould. As a result of these three factors, enzyme production proceeds at a permanent rate even in 70–80 hour old cultures. The mycelium of such cultures which are grown on starch-medium and have a poor production of amylase, after washing may be induced by renewed addition of starch.

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Address of the authors: ISTVÁN HORVÁTH, ATTILA SZENTIRMAI, SÁNDOR BAJUSZ, ÉVA PARRAGH
Research Institute of the Pharmaceutical Industry, Budapest VII. Rottenbiller u. 26.

CHARACTERIZATION OF ANTHRAX PHAGES

By

JUDITH LANTOS, I. VARGA and G. IVÁNOVICS

Institute of Microbiology, University Medical School, Szeged

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Summary. The characteristics of anthrax phages frequently present in soil samples were studied on 8 different strains. As a reference, W phage isolated from a lysogenic *B. cereus* W strain by McCLOY was used. The study covered the examination of plaque morphology, citrate sensitivity, adsorption, latency period and antigenic structure. The ultra-violet and heat sensitivity of the same strains was also studied.

On the basis of the above mentioned characteristics the nine strains were divided into 3 groups. The main differentiating feature was the antigenic structure, which was found to be in good parallelism with the other biological characteristics.

Part of the phages were subjected to lysogenization experiments. These attempts either failed, or, in some cases led to the formation of labile complexes between *B. anthracis* and the phage.

The term "A phages" is suggested to designate, the ubiquitous agents active against *B. anthracis*. As a prototype of these agents the W phage isolated by McCLOY is proposed. The present studies might offer basis for further grouping and systematology of A phages.

Using the methods recently developed in this laboratory, a high percentage of successful isolations of phages active against *B. anthracis* could be achieved. Out of some dozen phages isolated from different soil samples 8 strains have been characterized earlier according to their plaque morphology and host range [1]. These phage strains were found to be highly specific for *B. anthracis*. Against *B. cereus* the strains were active only if plain lysates or in some cases 1 to 100 dilutions were used. They had no effect whatever on other Gram negative organisms tested. Such a degree of specificity seemed to justify the designation of these agents as anthrax phages.

The phage strains isolated by McCLOY from a lysogenic *B. cereus* [2] and designated as W phage [3] was found highly specific for *B. anthracis*, while initiating lysis in a moderate number of *B. cereus* strains only. On the basis of these characteristics phage W may eventually be regarded as the prototype of anthrax phages.

The present report deals with experiments comparing the characteristics of our 8 recently isolated strains of anthrax phages to those of the W phage.

Materials and methods

Bacterium strains. Two strains of *B. anthracis* were used throughout. One of them was an asporogenic atypical strain of very moderate virulence (Davis strain), the other an acapsulogenic mutant of the Vollum strain. (As to the characteristics of the Davis strain, see McCLOY [2] and reports from this laboratory [4]). In some experiments a streptomycin-resistant mutant of the Davis strain was also used. All the Davis strains were kindly supplied by Dr. McCLOY.

The acapsulogenic mutant of the Vollum strain was obtained by the method of STERNE [5]. The mutant designed as Vollum-R proved to be avirulent for mice.

Phage strains. A detailed description of plaque-morphology of phage strains isolated from soil samples by the streptomycin method was published earlier [1]. Figures used for the designation of the strains refer to the protocol number of the corresponding soil samples.

W-phage was obtained by the courtesy of DR. McCLOY. The α -mutant of this strain was maintained by serial passages on Davis strain host cells.

The average particle content of the phage suspensions propagated on Davis strain was 10^8 to 10^{10} per ml. The lysates after being clarified by centrifugation were sterilized by the addition of chloroform and stored at -4° to -6° C until used.

The T2 coli phage was kindly supplied by DR. R. LATARJET (*Paris*). For the propagation and titration of this phage *E. coli* B strain was used.

Media. The YP (yeast extract peptone) medium was used throughout.

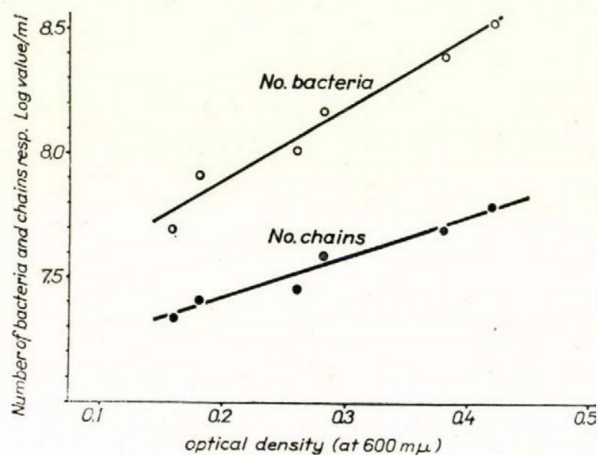


Fig. 1. Bacterial resp. chain count of *B. anthracis* Davis strain in fluid cultures at different optical density values

This fluid medium was converted into YP agar by the addition of 1.5 per cent agar-agar. On YP agar the phages developed plaques of typical morphology and the Vollum R-strain showed intense sporulation.

Determination of cell count in *B. anthracis* cultures. The varying grade of chain formation has been known to hinder quantitative work with *B. anthracis*. In rocked fluid medium cultures of the Davis strain the length of the chains exhibited remarkable variation according to the actual phase of growth, e. g. the average number of cells in a chain was 2.3 at the beginning of the logarithmic phase while 5.9 at its end. The number of cells per chain was determined optically in slide preparations fixed in BOUIN solution and stained as described by GUTSTEIN [7]. The chains were counted in a Bürker chamber. The optical density of growing cultures was measured as described earlier [6]. In Fig. 1 the relation of cell and chain count to the optical density is presented graphically for a logarithmically growing culture of the Davis strain.

The strain Vollum-R grew in long chains of a length varying from experiment to experiment; its cells were stouter than those of the Davis strain. In spite of these differences the turbidimetric estimation of cell count in Vollum-R strain cultures was always made on the ground of the extinction curve obtained for the Davis strain. This was done because of certain limitation in facilities.

Phage counting. The standard method of ADAMS [8] was used. Both phage and host cell dilutions were prepared in saline containing 20 per cent YP broth. To 0.5 ml of an appropriately diluted phage preparation 8×10^7 Davis strain cells were added in 0.5 ml volume. The phage-host cell mixture was then added to 1 ml melted YP agar (50° C) and layered over the surface of an agar plate. Phage counts were always made in duplicate.

To determine latency time 5 ml of a Davis strain culture of 0.3 optical density was infected with an appropriate phage-lysate. The multiplicity of infection was 1. The prewarmed

cultures were incubated at 37°C in a water bath under constant rocking at 10 cm amplitude and 100 rockings per minute. As indicated by preliminary experiments, samples were taken at 3 min. intervals.

Ultraviolet light source. A "Hanau" type germicide lamp was used with an energy emission of 25 erg/sec/mm² at 50 cm distance. Wave length was 2537 Å. Energy emission was measured by the photometer of LATARJET *et al* [9]. Under the experimental conditions used 200 erg/mm² energy was required for the 99 per cent inactivation of T2 phage. This result corresponds to those obtained by other authors [9].

Ultraviolet inactivation of phages. Crude lysates were diluted 1 : 100 with 0.9 per cent NaCl solution buffered to pH 7.3 by a mol/150 phosphate buffer. Five ml of the diluted phage material was carried into a Petri dish 10 cm in diameter and irradiation was performed under vigorous shaking.

Immune sera. Rabbits were immunized with 5 ml doses of phage lysate every 3rd day during a period of 10 weeks. Injections were given first subcutaneously, later intravenously.

In spite of repeated attempts, sera of a low antibody content were obtained on immunization by Wa phage.

Lysogenization. The selection of lysogenic strains was attempted from secondary colonies in plaques, from cultures surviving phage infection and sometimes from mixtures of phage and bacterium by inoculating onto the surface of YP agar plates. All such experiments were carried out with the Vollum-R strain. In the lysogenization experiments sporulation of bacteria was nearly complete after 3 to 4 days of incubation at 37°C on solid medium. The spores were resuspended in saline and washed twice by centrifugation. The washed sediment was thoroughly resuspended and fractionated by centrifugation. After run in a horizontal centrifuge for 10 minutes at 100 g, 95–98 per cent of the spores present in the supernatant were single. Aggregates of two or more spores were rare. The spore suspension was then heated to 85°C for 5 minutes, resulting in a 90 per cent loss of germinating capacity, and in a total inactivation of the phage particles present. The spore suspension thus obtained was kept in the refrigerator until used.

The proportion of spores carrying prophage was calculated from the results yielded by spreading a series of spore suspension dilutions 0.1 ml each onto the surface of YP-agar and by preparing soft-agar layers from mixtures of spore suspension and Davis strain cultures.

The lysogenic character of the suspected colony was proved by dropping its diluted suspension onto an indicator plate.

Results

Physiological characteristics of phages. The plaque morphology of the strains used in the present experiment was described earlier [1]. In this work phages with homogeneous plaque morphology were selected from all but one of the strains described above.

Three main morphological types of plaques could be differentiated when tested on Davis indicator strain. The clear plaques either great (*cg*) or small (*cs*) were the most readily recognizable. The second type of plaques was that described as target-like (*tl*), characterized by a pattern resulting from the different intensity of lysis within the single plaques. The entirely clear central area was surrounded by a partially lysed halo; the latter was separated from its surroundings by a thin, quite transparent border. Phage particles from *tl* plaques when propagated on Davis strain gave sometimes rise to mutants characterized by clear plaques. This phenomenon was regularly observed for phage 28 *tl* exhibiting a 5 per cent incidence of clear plaques in its progeny. It had to be supposed that this strain was a mixture of two different phage mutants and was therefore designated as 28 *tlcg*. Our other phage strains with *tl* plaques, *i. e.* strains 26 *tl* and 27 *tl* isolated from mixed cultures of phages

described earlier [1] proved to be homogeneous at the examination of thousands of plaques.

The third plaque-type was characterized by a diameter of 3 mm or more and a pattern consisting of a central clear area 0.5 to 0.7 mm in diameter, surrounded by a ring of full growth and an outer faint, loose border. This plaque type designated as *cr* (clear center with peripheral ring) was observed in two strains, *i. e.* 26 *cr* and 27 *cr*.

The strain termed in this paper 29 *cg* and selected from the 29 *cg* + *cr* culture described earlier [1] produced homogeneous *cg* plaques during serial transfers.

A plaque morphology very similar to that of the Davis strain was demonstrated by the Vollum-R strain used as an indicator, with a somewhat less distinct pattern, the Vollum-R strain not being lysed very readily. The efficiency of plating of the phage materials was essentially similar for both indicator strains. This result together with the citrate sensitivity and latency time of the corresponding phage preparations is presented in Table I.

Table I
Some characteristics of the phages used

Phage	Plaque morphology	E. O. P.*			Latency time (minutes)
		Davis	Davis sodium citrate**	Vollum-R	
26 <i>cs</i>	clear, small	1	1	1	28
28 <i>cg</i>	clear, great	1	1	1	30
29 <i>cg</i>	clear, great	1	1	1	28
26 <i>tl</i>	target like	1	1	1	40
27 <i>tl</i>	target like	1	1	1	30
28 <i>tlcg</i>	target like, clear, great	1	1	1	25-30
26 <i>cr</i>	clear, with peripheral ring	1	0.7	0.2	30
27 <i>cr</i>	clear, with peripheral ring	1	0.34	0.3	26-28
W <i>a</i>	clear, great	1	1	0.5	28

* Efficiency of plating.

** In presence of 1 per cent sodium citrate.

The latency period was within 25 to 40 minutes for all the strains. A moderate citrate sensitivity of strains 26 *cr* and 27 *cr* was observed.

The adsorption of phages onto Davis strain suspensions of 3×10^7 to 10^8 cells per ml was examined at the 10th and 25th minute of incubation at 37° C. The results are summarized in Table II.

Adsorption was rapid. More than 90 per cent of all but 3 strains was adsorbed within 25 minutes under the experimental conditions used. Some-

Table II
Adsorption of phage strains to Davis bacteria

Phage	Cell count per ml	Multiplicity	Per cent of adsorption*	
			10 minutes	25 minutes
26 <i>cs</i>	1×10^8	1.0	87	94
28 <i>cg</i>	3×10^7	0.12	82	98
29 <i>cg</i>	3×10^7	0.7	89	99.7
	1×10^8	0.4	89	97
26 <i>tl</i>	3×10^7	0.3	87	92
	1×10^8	0.1	89	92
27 <i>tl</i>	3×10^7	0.2	90	96
	1×10^8	1.0	88	97
28 <i>tlcg</i>	3×10^7	0.17	56	67
	3×10^7	0.17	76	85
26 <i>cr</i>	1×10^8	0.8	60	81
	1×10^8	0.4	63	75
27 <i>cr</i>	3×10^7	0.2	59	77
	3×10^7	0.7	60	68
W <i>a</i>	3×10^8	0.1		95

* To 1 ml of YP bouillon cultivated Davis strain of 0.2 to 0.3 optical density 1 ml of the appropriately diluted phage material was added. Out of this mixture 0.1 ml was added to 9.9 ml of ice-cooled dilution fluid after 10 and 25 minutes, respectively, and centrifuged until cold. The phage count was determined in the supernatant.

what different results were obtained in two similarly but separately performed experiments with 28 *tlcg* phage. Adsorption of this phage was somewhat slower than the average of the others.

Heat sensitivity of the phages. An equal amount of lysate was added to 1 ml of pH 7.3 phosphate buffer preheated to the temperature required in a water bath. The dilution of the lysates was so chosen that the final mixture should contain 10^7 to 10^8 phage particles per ml. Most of the phages studied were remarkably heat-labile at 60° C. The high rate of inactivation made it impossible to follow its dynamics. For this reason only inactivation curves obtained for different phages at 56° C are demonstrated in Fig. 2.

Out of the 5 curves in Fig. 2 one represents the inactivation of 28 *cg*, 28 *tlcg*, 26 *cs* and 29 *cg* phages; the results obtained with these agents were identical. Another curve is valid for both 26 *cr* and 27 *cr* phages, these having dynamics of inactivation essentially similar within the limits of the experimental errors.

All the inactivation curves have a common shape, *i. e.* an initial straight line successively changing to a slight slope. This type of inactivation curve was described earlier also for the tempered megaterium [10] and pyocyanus phages [11].

The cause of this particular shape of the inactivation curve was not investigated. Both the presence of variants in a certain phage population and the existence of heat resistant genotypic mutants may well be supposed.

Wa phage exhibited a remarkable heat resistance; a certain correlation was observed between plaque morphology and heat sensitivity. Strains *26 cr* and *27 cr* were of medium sensitivity, while strains with *tl* or clear plaques were highly sensitive to heat and especially so the strains producing purely *tl* plaques.

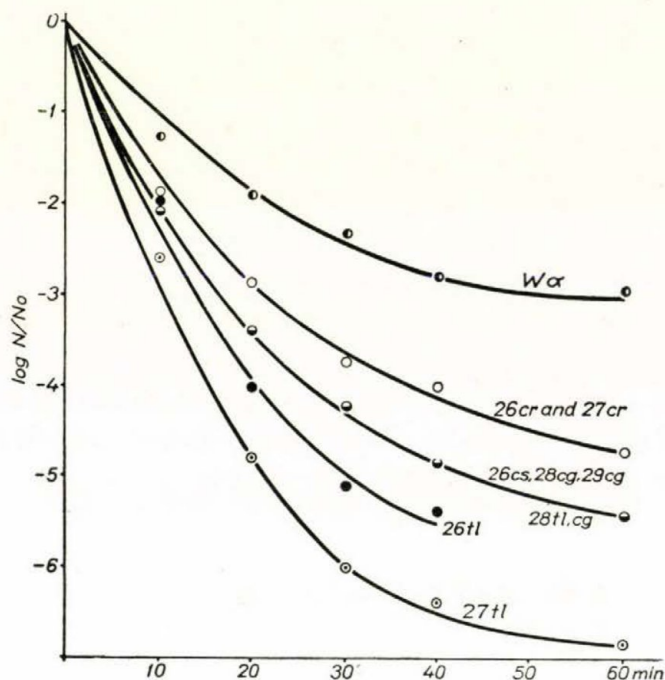


Fig. 2. Heat inactivation of different phages at 56° C

Sensitivity of the phage strains to UV irradiation. When presented graphically in a semilogarithmic coordinate, the initial phase of the UV inactivation curve followed a first order reaction rate. A gradual sloping of the curve occurred when the amount of survivors decreased by 4 to 5 orders of magnitude. The only exceptions were strains *26 cr* and *27 cr*; both were inactivated at a first order reaction rate. The two main types of inactivation curves are presented in Fig. 3.

The UV inactivation curve of T2 coli phage as compared to those of some anthrax phages are demonstrated in Fig. 4. In this figure only the straight-line phases of the inactivation curves are presented.

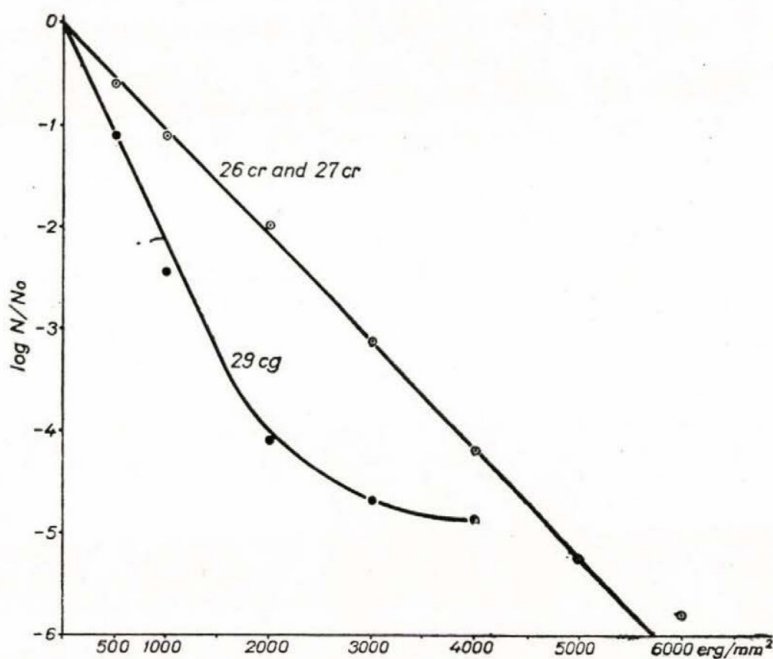
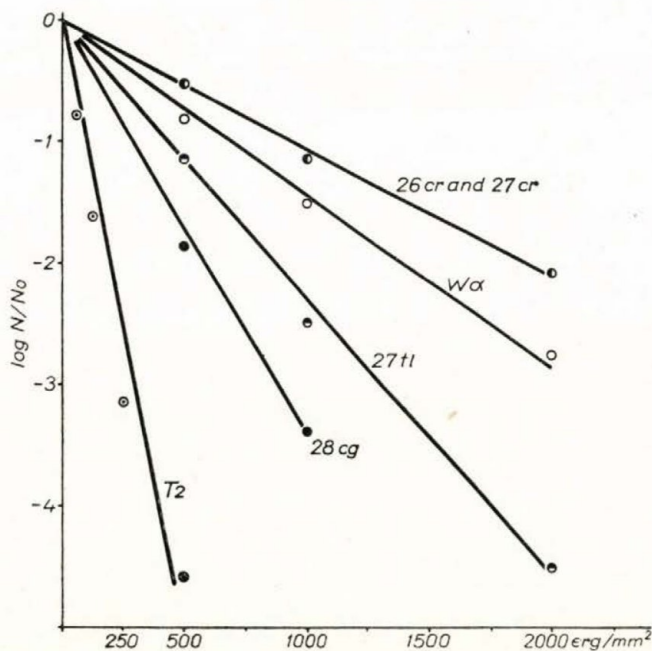


Fig. 3. UV inactivation of 26 cr and 29 cg phages

Fig. 4. UV inactivation of *E. coli* T₂ and different anthrax phages

The numerical relations of UV sensitivity of the single phage strains are given as the amount of energy necessary for the inactivation of 99 per cent of the particles (see *Table III*).

Table III

Energy required for 99 per cent inactivation of phages by UV irradiation as interpolated from experimentally determined data

UV dose erg/mm ²	Infective particle count of the phage strains (log)*									
	T ₂	26 cs	28 cg	29 cg	26 tl	27 tl	28 tlcg	Wa	26 cr	27 cr
0	8.15	8.45	7.89	7.18	8.22	8.90	8.91	8.26	7.78	7.75
62.5	7.48									
125	6.52									
250	4.9									
500	3.58	6.61	6.04	6.08	7.15	7.81	7.69	7.43	7.27	7.28
1000		5.07	4.50	4.74	6.15	6.13	6.30	6.73	6.72	6.66
2000		3.71	3.4	3.84		4.00	5.15	5.46	5.61	5.76
Energy required for 99 per cent inactivation	200	650	630	775	1000	875	875	1400	1875	1875

* Means of 2 to 3 experiments.

Antigenic structures of phage strains. The analysis was carried out with immune sera against 3 selected phage strains. The neutralizing velocity constant of the anti-Wa serum was very low. The speed of inactivation was expressed with the velocity constant and calculated from the formula:

$$K = 2.3 \frac{D}{t} \log \frac{P_0}{P}$$

suggested by ADAMS [8].

Three antigenically different groups were revealed by the serological analysis of the 9 phage strains tested. Phages isolated from soil samples Nos. 26, 27 and 29 and giving rise to *tl* or clear plaques belonged to one serological group. Strains with *cr* type plaques isolated from soil samples Nos. 26 and 27 formed another serologically distinct group. Phage Wa had an antigenic structure different from those isolated in this laboratory. Nevertheless some serological relations between Wa and 27 *cr* phages seemed to be demonstrable. Serological analysis may thus serve as a means of grouping anthrax phages.

Virulence of phages. Part of our phage strains seemed to be virulent; they brought about total lysis resulting in the formation of clear plaques. Therefore no attempt was made to lysogenize *B. anthracis* by any of the strains. On the other hand the *cr* and *tl* character of the plaques formed sug-

Table IV

K values for anti-28 *tlcg*, 27 *cr* and *Wa* immune sera obtained in experiments at 37° C

Phage	K values from individual experiments and their means		
	anti-28 <i>tlcg</i> ⁽¹⁾	anti-27 <i>cr</i> ⁽²⁾	anti-W3 ⁽³⁾
26 <i>cs</i>	59 68.5 78	0.0	0.0
28 <i>cg</i>	67 71.3 77 70	0.0	0.0
29 <i>cg</i>	97 88.3 67 101	0.0	0.0
26 <i>tl</i>	115 106.0 97	0.0	0.0
27 <i>tl</i>	69 74.5 80	0.0	0.0
28 <i>tlcg</i>	75 97.2 115 87 111 98	0.0	0.0
26 <i>cr</i>	0.0	233 225.5 212	0.0
27 <i>cr</i>	0.0	209 215.0 221	3.0
<i>Wa</i>	0.0	10.0	17.0

(1) Diluted to 1:100 to 1:500.

(2) Diluted to 1:100 to 1:1000.

(3) Diluted to 1:10 times of sampling were the 5 th, 10 th, 20 th, and 40 th, minutes of the experiment. Sign 0.0 means no effect whatever within 40 minutes even at the lowest serum dilution used.

gested the corresponding phages to be tempered, thus strains 26 *cr*, 27 *cr*, 26 *tl* and 27 *tl* seemed to be agents appropriate for lysogenization.

Infection with the latter four strains resulted in a rapid lysis with no secondary growth of Davis strain cultures in fluid medium. On the other hand the slow and incomplete lysis usually followed by a secondary growth suggested the Vollum strain to be suitable for lysogenization.

In our numerous attempts to obtain stable lysogenic isolates mostly the phage strains 26 *cr* and 27 *cr* were used. Though these attempts have failed, there was a high incidence of labile lysogenic complexes. One of our experiments is presented below. An exponentially growing Vollum-R culture was infected with phage 26 *cr* (multiplicity 1) and reincubated for 6 hours. After this period lysis was usually advanced. Samples from such cultures were spread on YP agar and incubated for 3 days at 37° C. The well sporulated

cultures were now suspended and heat-treated as described above. An 0.1 ml inoculum of a 10^{-4} dilution of the spore suspension thus obtained yielded 180 Vollum-R colonies when carried onto the surface of a YP agar plate.

The incidence of plaque forming agents in the spores was examined by mixing 0.1 ml spore suspension to Davis and streptomycin resistant Davis strains; the mixtures were made up to a soft agar overlay on YP and streptomycin containing (40 $\mu\text{g/ml}$) agar plates, respectively. No plaques were formed on the streptomycin containing plates, while 90 were formed on YP medium, i. e. 50 per cent of the germinating spores contained the plaque forming agent. The plaques exhibited the characteristic morphology. After 48–72 hours of incubation central secondary growth sometimes became evident. Bacteria from these central colonies were reisolated on YP agar medium and incubated until sporulation was complete. The suspended spores were then heat-treated to kill not only the phage present but also the Davis cells contaminating the suspension by chance. As to the incidence of plaque forming agents in the spores, individual experiment yielded 30 to 50 per cent.

Using different methods of lysogenization, results essentially similar to those just described were obtained, with a decreasing incidence.

Similar experiments carried out with phages of the *tl* plaque type (strains 26 *tl* and 27 *tl*) have failed; not even the formation of the loose prophage-spore complex described above could be demonstrated.

Discussion

A remarkable advance in the knowledge about anthrax phages have been the recent studies by McCLOY [2, 3]. She isolated from a lysogenic strain of *B. cereus* a phage highly specific for *B. anthracis*. The specificity of the phage was further increased by propagating it on Davis strain host cells. The phage released by the lysogenic *B. cereus* strain had two mutants designated α and β . The β phage being usually dominant in the progeny, is temperate and highly specific for *B. anthracis*, while the α mutant is more virulent and less specific [12].

The new method developed in this laboratory [1] has made it possible to isolate from soil samples both the actually free phage particles and also those released by lysogeny. Streptomycin resistant cells of *B. anthracis* grown in the presence of the antibiotic served for the enrichment of the phages present in the sample. Naturally the possibility of selection of certain mutants or even strains and also the modification of the phage population during this primary cultivation cannot be excluded.

The phage strains examined in the present study may be designated as anthrax phages on the basis of their host specificity. These phages are common

in nature [1]. We suggest for the general designation of all the phages active against *B. anthracis* the group name "A phage". From the taxonomical point of view this has the advantage of not involving any obligations that may interfere with any later systematology of these agents.

On the basis of their antigenic structure the 8 strains isolated in this laboratory and the W strain of McCLOY were divided into 3 separate groups. This grouping seems to correspond to biological characteristics other than antigenic as well.

Table V

Grouping of A phages on grounds of their antigenic structure and other biological characteristics

Antigenic structure (group)	UV sensitivity ¹⁾ (erg/mm ²)	Citrate sensitivity ²⁾	Heat resistance	Plaque morphology	Phages belonging to the group
A ₁	1400	—	great	clear or target like ³⁾	W
A ₂	1875	+	medium	clear center with peripheral ring	26 <i>cr</i> ; 27 <i>cr</i>
A ₃	630—1000	—	little	clear or target like	26 <i>cs</i> ; 26 <i>tl</i> ; 27 <i>tl</i> ; 28 <i>tlcg</i> ; 28 <i>cg</i> ; 29 <i>cg</i>

(1) Energy needed for 99 per cent inactivation.

(2) In the presence of 1 per cent sodium citrate.

(3) α and β mutants, respectively. According to McCLOY [3] both of the mutants have an identical antigenic structure.

Group A₁ is represented by the W phage strain, the prototype of A phages. This strain could be sharply differentiated from the others by serological methods, though a certain overlapping with strain 27 *cr* belonging to group A₂ was regularly observed. The A₁ group phages are further characterized by a high heat tolerance and a medium ultraviolet sensitivity. Strains 26 *cr* and 27 *cr* belong to group A₂ and are characterized by their plaque morphology, specific antigenic structure, high ultraviolet tolerance and moderate citrate sensitivity. Group A₃ differs from the other strains with the target-like or clear plaques, common antigenic structure and being highly sensitive to both thermal and ultraviolet influences.

We are aware that the present grouping can by no means be regarded as definite. Still we think that it might serve as preliminary basis for a systematology of the anthrax phages. Plaque morphology, owing to the high probability of mutations, does not seem to be of decisive importance but is still of use in recognizing members of the A₂ group.

The nature of the earlier described strain 29 *cr* + *cg* remains unsettled. This strain was a mixture of particles inducing the formation of clear great, and *cr* plaques. In the present work only strain 29 *cg* was examined, which

had been selected from an appropriate isolated plaque originating from the initial strain. As the phage strain forming *cr* plaques has been lost, it is no more possible to decide whether the original strain was a mixture or a mutual ancestor of the two mutants isolated.

As regards the virulence of our phage strains, we have been unable to form a definite opinion. In the case of anthrax phages virulence appears to be host dependent. McCLOY did not succeed in lysogenizing the Davis strain with *Wa* phage [2], while this was possible by means of sporogenic strains. She observed that "when a sporing strain was exposed to phage *a*, lysogenic colonies were obtained; but they were lysogenic with β , not with *a* itself" (McCLOY, personal communication). We succeeded in lysogenizing the Vol-lum-R strain with *Wa* phage, and could demonstrate the progeny to consist purely of *a* particles (IVÁNOVICS, to be published). Altogether the difficulties of examining the problem of virulence of anthrax phages appearing much greater than usual. As LWOFF pointed out in his excellent monography "Unfortunately, the definition of the character virulent is purely negative... Thus, as a result of the study of a system with low lysogenization quotient, a temperate phage could be considered as virulent".

We succeeded in obtaining prophage carrying spores using phages from the A_2 group, while we failed in our attempts to keep them in a purely lysogenic state. The very high incidence of non-lysogenic segregants observed is a feature very uncommon with lysogenic strains. This might have been due to the technique used, heat-treatment being probable deleterious for prophage carrying spores. Were this the actual situation, it would explain the shift in the proportion in favour of non-lysogenic spores.

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Address of the authors: JUDIT LANTOS, ISTVÁN VARGA, GYÖRGY IVÁNOVICS
Institute of Microbiology, University Medical School, Szeged, Beloiannisz tér 10.

"PROTOPLAST" PRODUCTION INDUCED BY PENICILLIN FROM THE CELLS OF *B. CEREUS* AND *B. ANTHRACIS*

By

J. FÖLDES and KATHLEEN MERÉTEY

Institute of Microbiology, University Medical School, Szeged

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Summary. Protoplast production was induced with penicillin in the penicillinase producing strain *B. cereus* NRRL 569. Conditions and optimum circumstances of induction have been discussed. The developed "protoplasts" do not adsorb phages and are incapable of reproduction. Therefore they may be considered as true protoplasts.

In a suitable environment, bacteria deprived of their cell wall develop into spherical bodies. The protoplasma is held together by the "cytoplasm membrane". These forms surrounded by osmotic barriers are capable of almost all the activities of the living cell, except reproduction and the adsorption of phages. Such forms originating from Gram-positive bacilli and cocci are termed bacterial protoplasts [1].

The method generally employed for producing protoplasts is hydrolysis of the cellular wall with lysozyme [2, 3]. *B. megaterium* and *B. subtilis* have proved most suitable for such studies. It is, however, striking that *B. anthracis* and *B. cereus*, both aerobic and sporeforming organism resistant to lysozyme, should not produce protoplasts upon treatment with lysozyme. Some investigators therefore attempted to induce protoplast production by other methods. STÄHELIN [4] suspended *B. anthracis* cells in Ringer's solution, but "plasmolysis" occurred in not more than two strains out of twenty. From the spore wall of *B. cereus*, STRANGE and DARK [5] produced an enzyme which hydrolyses the cell wall. This extract was found to be species specific. We ourselves succeeded to induce by means of pretreatment with penicillin satisfactory protoplast production by *B. cereus* as well as by *B. anthracis*. Since in the case of *B. cereus* phosphatase production [6] is accompanied by penicillinase synthesis, these microorganism has been chosen for our detailed studies.

Some theoretical questions are associated with the problems of methodology. Investigation into the development of protoplasts, the production of phosphatase and the synthesis of penicillinase provides possibilities for studying not only the mechanism of protoplast production, but also the mode of action of penicillin. The present report deals with some methodological aspects of protoplast production; the results obtained by investigation of the above mentioned theoretical questions will be discussed elsewhere.

Materials and methods

Bacterial strains. The strains *B. cereus* NRRL 569 and NP-anthrax [7] were employed.

Phage material. Following the method of IVÁNOVICS and LANTOS [8], we isolated different phages from various samples of soil of those propagated on NRRL 569, phage "Ca 569" [9] was used in the experiments.

Culture media. Two different kinds of medium were employed. Medium YP contained the supernatant of autoclaved yeast and Witte's peptone [10]. The basic synthetic medium prepared after the description of WIAME and STORCK [11], was completed with yeast extract and casein hydrolysate [12].

Stabilizing medium. As stock solution a 2.4 M sucrose solution was employed; it was added to the culture after dilution with M/15 phosphate buffer at the desired ratio.

Penicillin stock solution. 10 000 I. U. penicillin G per ml was dissolved in physiological saline or in 1 M MgSO_4 .

Determination of penicillin was carried out by the well known "cup-plate" method. *Staphylococcus aureus* (Walker) strains served as indicator strain.

Determination of penicillinase was performed by the microbiological method of POLLOCK [13]. As substrate, 20 I. U. of penicillin per ml were used in YP broth containing 1 per cent of gelatine. To 8 ml of this we added 2 ml of the supernatant containing penicillinase. Of the mixture, incubated at 30° C, a sample of 1 ml was taken every fifteen minutes and added to 9 ml of 0.01 M ice-cold potassium phosphate buffer of pH 7. In this mixture the amount of non hydrolysed penicillin was determined by the above mentioned "cup-plate" method.

Results

Production of protoplasts. The *B. cereus* cultures of varying age were not optimal for the production of protoplasts when the inoculum was an old culture (for instance a twelve hours old slant agar or broth culture). However, inoculation with growing cells in the log phase (3 to 4 hours old culture) resulted an optimum in the development of protoplasts. Consequently we acted as follows. YP broth inoculated with the spore suspension of *B. cereus* NRRL 569 was incubated at 30° C with aeration on a reciprocating shaker. In the logarithmic phase of growth (0.30 and 0.40 optical density, in the following OD) 0.1 ml of the culture was transferred to 10 ml of fresh YP broth and cultivated there in the described manner. For cultivation an Erlenmeyer flask fitted with side-tube was used [14]; optical density of the cell suspension was measured with a densitometer. At the desired OD, 0.15 ml of the penicillin stock solution and 5 ml of the stabilizing sugar solution were added to the culture. Incubation of the cell suspension thus treated was continued for fifteen minutes under aeration, then for two to three hours without aeration. Transformation of the cells was studied under a phase-contrast microscope with a magnification of 600. The protoplasts obtained by this procedure are shown in Fig. 1.

The protoplasts appear under the microscope as a spherical dark and compact bodies. With oil-immersion, some cells are found to contain granules. In some instances rods are seen among the protoplasts.

Optimum conditions for the production of protoplasts. Owing to the nature of the material, protoplast transformation cannot be followed closely by quantitative assay. The optimum conditions of transformation were established

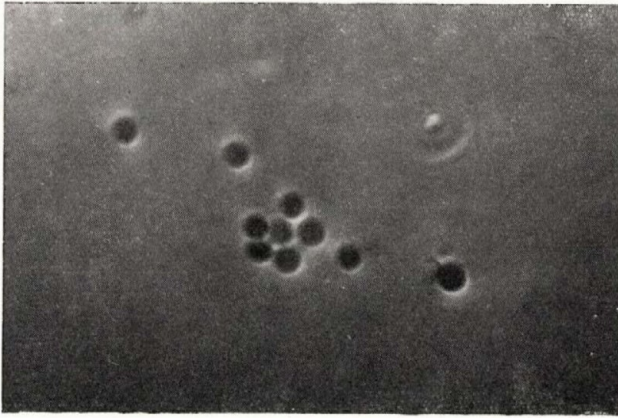


Fig. 1. Penicillin induced protoplasts

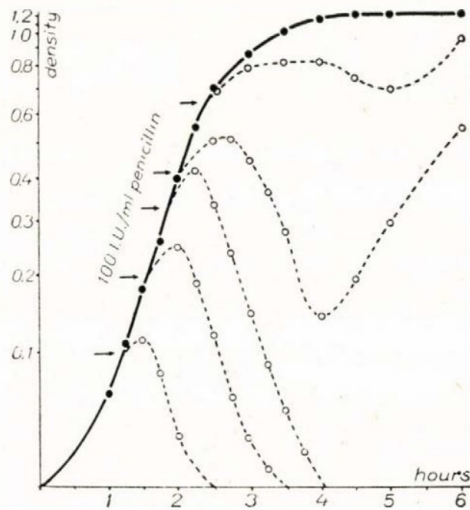


Fig. 2. The effect of penicillin on the growing cells of *B. cereus* 569

on the basis of the protoplast's morphology, stability tests and counts. Tests for stability were made on protoplasts kept in a refrigerator for 12, 24 or 48 hours.

Cellular transformation is greatly influenced by *the age of the cell culture*. The effect of the penicillin on the growing cells of *B. cereus* depends on the period of growth. This penicillin action is represented in Fig. 2 by the values referring to the change of density.

As illustrated in Fig. 2, penicillin brings lysis of the cells and clearing up of the cell suspension when added to the culture in the first phase of loga-

rhythmic growth; later penicillin elicits only partial lysis followed by renewed reproduction of the cells.

If the stabilizing sucrose solution is added to the culture simultaneously with the penicillin, protoplast formation follows. Complete development into protoplasts, however, occurs only in young cultures that are cleared up perfectly on the action of penicillin without the stabilizing sugar solution.

To determine the optimum amount of penicillin, 5, 10, 50, 100 or 500 I. U. per ml were added to cell suspensions of identical age and the conditions of transformation were studied in the presence of stabilizing sugar solution. Protoplast production was induced in an equal measure by amounts of penicillin over 10 I. U. per ml; as revealed by microscopic investigation, cellular transformation was about complete in every case (50 to 500 I. U. per ml penicillin). Taking into consideration the intense synthesis of penicillinase, further 100 I. U. penicillin per ml were added to the cells.

Mg^{++} was found to play an important role in stabilizing the protoplasts. Protoplast suspensions prepared without Mg^{++} and with 0.01 M $MgSO_4$ were stored at 4° C for two days. In the presence of Mg^{++} , the protoplasts retained their size, remained intact, and did not change in number. Protoplasts prepared in the absence of Mg^{++} decreased in number, the remaining forms shrank and became granular.

Determination of the optimum sugar concentration. 100 I. U. of penicillin per ml and 0.01 M $MgSO_4$ were added to the cell cultures of 0.3 to 0.4 OD, while sugar concentration was varied between 0.2 and 1 M. The developed protoplasts were tested for stability by the above mentioned method. Sugar concentrations of 0.7, 0.8 and 0.9 M were found the most appropriate. Amounts of 0.8 M were hence employed as stabilizing medium in subsequent experiments.

Effect of sugar solution on cell suspension. The comparatively high concentration of sucrose gave rise to noteworthy changes in the density of the cell suspension.

Fig. 3 demonstrates that sucrose solutions added at various points of time to growing bacterial cultures immediately caused a very marked decrease of density. Cell structure was left unchanged, but chains with 2 to 4 members were broken up into separate units. Reproduction of the bacterial cells continued after the addition of sugar; germ counts performed in the sixth hour of the cultivation revealed the presence of equal number of colony-forming units (7.6×10^8) in the cultures. It is remarkable that in the control culture the germ count was of a similar order of magnitude, in spite of the higher optical density.

pH changes in the environment exert a strong influence on protoplast production. This effect was studied on synthetic media. A medium of twofold concentration was diluted in equal proportion with M/15 phosphate buffer with a pH varying between 5 and 8. Cells grown in YP broth and washed

in salt water were suspended in this buffered medium; after addition of the required amount of penicillin, MgSO_4 and sucrose, the suspension was incubated at 30°C . Transformation was controlled after four and twenty-four hours. The most suitable range was between pH 6 and 7.5; subsequently the media were adjusted to that range. In the acid range (pH 5 to 6.5) a considerable per cent of the bacilli failed to develop into protoplasts.

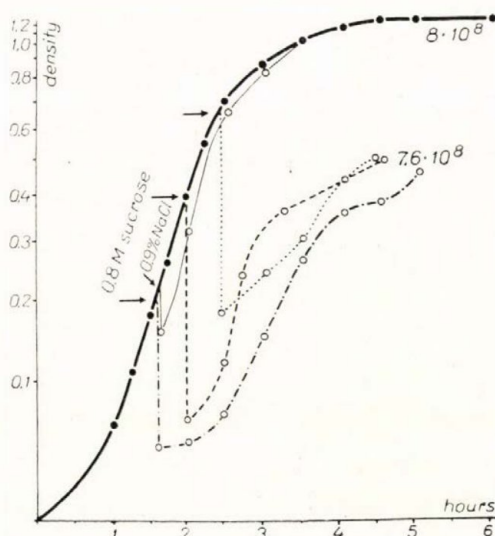


Fig. 3. The effect of 0.8 M sucrose on the growing cells of *B. cereus* 569

Quantitative aspects of protoplast production. As confirmed by the curves presented in Fig. 2 only in the first half of the logarithmic phase were the cells capable of developing into protoplasts. This capacity of young cells gradually diminished in the course of growth, and with time more and more intact bacterial cells could be distinguished among the protoplasts. A few intact bacilli were found even in apparently completely transformed suspension; their number and ratio were established by germ counts. The results revealed that of each million cells, one or two colony forming cells remain in an intact condition capable of reproduction.

The amount of the penicillin added to the cell suspension gradually decreased as protoplast formation was proceeding; at the same time penicillinase could be demonstrated in the medium. In the first fifteen minutes of incubation the 100 I. U. penicillin per ml were reduced to 1 I. U.; in sixty minutes even this was completely hydrolysed.

Tests concerning specific phage adsorption and the quantitative investigation of adsorption were carried out according to ADAMS' [15] standard method.

The protoplasts were centrifuged and the particles were suspended in YP broth containing 0.8 *M* sucrose. Infection of higher and lower multiplicity (1 and 0.01) with phages was performed, and the rate of adsorption was determined after twenty minutes incubation. In the control phage suspension, the number of particles was on the basis of three parallel counts, 1.2×10^8 ; after adsorption this value varied between 0.9 and 1.1×10^8 .

By means of the method described, protoplasts were produced also from the NP-Anthrax strain. Although *B. anthracis* does not produce penicillinase, curves reflecting the effect of penicillin displayed strikingly the same character as did the curves of *B. cereus* in Fig. 2. However, in the case of *B. anthracis* larger amounts (500 I. U. per ml) of penicillin were required, while 0.5 *M* sucrose sufficed for stabilization. As regards of the protoplasts' production the results of the experiments with *B. anthracis* were the same as those recorded with *B. cereus*. Concerning the principle of the mode of action of penicillin, our attention was drawn in the first place to *B. cereus* which produces penicillinase; no further experiments were consequently undertaken with the protoplasts of *B. anthracis*.

Discussion

For the production of protoplasts penicillin is generally used in the case of Gram-negative bacteria [16, 17]. Osmotically fragile cells were obtained from *E. coli*, but several authors refuse to accept them as real protoplasts [18, 1]. This protoplast-like bodies bear remnants of cell walls, adsorb phages, and even possess the capacity of forming colonies in culture media. WEIDEL and PRIMOSIGH [19] separated the two layers of the cellular wall of *E. coli* B by a chemical procedure. The outer layer proved to consist of lipoprotein, the inner one of lipopolysaccharide. This double layer was clearly demonstrated electronmicroscopically by KELLENBERGER and RYTER [20]. The inner layer maintains the structure of the cell and ensures the rigidity of the cell wall; it is chemically similar to the cell wall of Gram-positive bacteria.

The mode action of penicillin has not been elucidated. PARK and STROMINGER [21] have shown that penicillin inhibits the cell wall synthesis in *Staphylococcus aureus* by blocking the enzyme performing transglycosidation processes. PRESTIDGE and PARDEE [22], on the other hand, believe that penicillin induces an enzyme which hydrolyses the cell wall, thus bringing about lysis of the cells.

In Gram-negative microorganisms the effect of penicillin is confined to the inner mucopolysaccharide part of the double-layer cell wall. Layers so widely different in composition cannot be found in the cell wall of Gram-positive bacteria. Consequently the whole of the cell wall disappears on the

action of penicillin and the arising spherical bodies become incapable of both phage adsorption and reproduction.

The osmotically fragile spherical bodies developed from *B. anthracis* are regarded by WEIBULL [16] as true protoplasts. Partly on the basis of the above reasoning, and partly in view of the absence of phage adsorption and the capacity for proliferation, the “protoplasts” of *B. cereus* — phylogenetically so close to *B. anthracis* [6] — may be also accepted as true protoplasts.

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Address of the authors: JÓZSEF FÖLDES, KATALIN MERÉTEY
Institute of Microbiology, University Medical School, Szeged, Beloiannisz tér 10.

THE DISSOCIATION OF SHIGELLA SONNEI ON A CULTURE MEDIUM CONTAINING DESOXYCHOLIC ACID

By

B. SERÉNY

State Institute of Hygiene, Budapest

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Summary. (i) The preparation of a new medium, D agar, has been described.

(ii) On D agar *Sh. sonnei* shows considerable dissociation. On the basis of colonial morphology phase I and II colonies and the *R* form can be distinguished from each other.

(iii) The reliable identification of *Sh. sonnei* strains can be performed by means of cultivation on yeast extract agar and on D agar.

In a previous report [5] a reliable new method has been described for the morphological differentiation of agar plate colonies of *Sh. sonnei* containing phase I, phase II or *R* antigens. In the present work the dissociation forms of *Sh. sonnei* cultures developing on a new medium containing desoxycholic acid will be discussed.

Materials and methods

D agar. For differentiating the various colony forms of *Sh. sonnei*, in addition to nutrient agar prepared with yeast extract, a new medium containing desoxycholic acid was employed (D agar). This modified form of MACCONKEY's agar was prepared as follows.

(1) Agar base. 170 g agar added to 5 l distilled water and autoclaved at 120° C for 30 min.

(2) 200 g Richter peptone, 50 g NaCl and 100 g lactose dissolved by heating in 5 l distilled water. To the solution 15 g desoxycholic acid (Richter) dissolved in 70 ml warm *N* NaOH is added. The warm agar base is mixed with solution 2 (also warm) and the pH is adjusted to 7.4. Then the medium is distributed in flasks at 1 l amounts and autoclaved at 115° C for 25 min. After autoclaving the medium is densely turbid. This medium base can be stored for 3 weeks in the refrigerator at 4° C.

For preparing plates the medium base is melted in the Arnold apparatus, filtered through cotton wool and to each litre 1 ml 1 per cent aqueous neutral red solution is added. The medium is then poured in sterile Petri dishes at 20 ml amounts. The plates can be stored for two weeks in the refrigerator.

The examination of colonies in oblique light and other methods employed have been described previously [5]. The importance of the proper technique of streaking should again be stressed. The examination by our method can only be performed when there are many separate colonies, 5 to 10 mm apart. The incubation period is 1 day at 37° C.

Apparatus for measuring the angle of incidence (position of plates). Using the apparatus devised by P. HEGEDŰS, mechanic, *State Institute of Hygiene, Budapest*, allowed the culture plate to be fixed in the position required, so that angle of incidence of the light was easily determined.

Results

Dissociation forms of Sh. sonnei. As in our earlier classification, *Sh. sonnei* colonies developing on D agar were divided into 3 groups (Table I).

Table I
The colonial appearance of Sh. sonnei on D agar

Group	Type	Morphological characters	Agglutinability
1	a	S form or similar colonies with brown star formation	phase I + II
	b	Bombshell form colonies with brown star formation	phase I + II
	c	R form colonies with brown star formation	phase I + II
2	a	S form colonies, brownish at "glistening" plate positions	phase I
	b	R form or irregular colonies without brown star formation	phase II
	c	Flat, less opaque, R form or irregular colonies without brown star formation	phase II
3		Margin of colonies reticulated at "glistening" plate positions	R (sometimes phase I)

Cultures shown by slide agglutination in absorbed sera to contain both phase I and phase II antigens were classified into *group 1*. These cultures may be regarded as serologically equivalent with colonies producing star formation on yeast extract agar. They grow in variable colony forms and can be differentiated into subgroups only with certain arbitrariness.

a) S form colonies or colonies resembling the S form. They are pinhead sized or somewhat larger. At reflected light they are whitish or yellowish-white, convex, with a smooth and moist glistening surface.

b) Colonies of bombshell-shaped appearance. They are 1 to 4 mm in diameter. This type differs from the former one in the following respects. A smaller or larger area of the S form colony is flat, more translucent, has an irregular surface and an undulated margin.

c) The R form colonies are 1 to 5 mm in diameter, irregular in shape and whitish in colour; have an undulated or lobated margin and a somewhat irregular surface. In direct light the flat colonies are less turbid than forms belonging to group 1*a*.

Colonies belonging to group 1 appear in oblique light as follows. (Plate position 2—4, distance from the light source 15 to 25 cm; e.g. between angles of incidence 65° and 75° at 15 cm distance.) The central part of the colony consists of a smaller or larger yellowish brown or distinctly brown sharply limited irregular area with a more or less crenated edge. This star-like formation which is sharply contrasted to the whitish surroundings, was called the "brown star". It should, however, be mentioned that the "star" sometimes resembles a circular or oval body. The brown stars develop after 16 to 24 hours of incubation; on keeping the plates at room temperature for some days they become more distinct; then after 4 to 8 days they slowly turn indistinguishable.

In natural transmitted light the brown stars are generally more clearly visible than in artificial illumination.

According to slide agglutination in absorbed sera, the brown star itself contains phase I antigen, while the culture constituting its whitish surrounding area consists of phase II antigen. The identical antigenic composition of star colonies grown on yeast extract agar and group 1 colonies cultured on D agar can be confirmed by cross-subcultures.

On D agar chiefly bombshell-type (*1b*) colonies are formed by freshly isolated *Sh. sonnei* strains (*i. e.* first subculture of strains isolated on D. C. agar from faeces or cultures obtained from the eye-discharge of guinea-pigs with *keratoconjunctivitis shigellosa*). Storing the S antigen-containing strains on DORSET's medium in the refrigerator (3–8° C) and streaking them from time to time onto D agar, depending on the individual properties of the strains, phase I (*i. e.* colonies with brown star) can generally be observed for 4 to 6 months. This form may sometimes be present for as long as two years. The longer the storage, the lower the incidence of such colonies. On agar slant cultures this decrease takes place more rapidly. According to the author's experience, phase I can be preserved in a culture for long periods if the Dorset culture is at 2 or 3 month intervals streaked on yeast extract or D agar and one colony showing star formation is transferred to DORSET's medium.

Group 2 contains colony forms showing in slide agglutination the presence of only one antigen.

a) S colonies, 0.5 to 1 mm in diameter. In direct light they are whitish, opaque, circular, with a smooth, glistening surface and a sharp edge. In oblique illumination at plate position 4–5 the periphery of the yellowish-brown or brown colony contains a very narrow, indistinctly limited, blurred whitish margin. This form is agglutinable only in *Sh. sonnei* phase I serum. It corresponds to the highly refractive homogenous colonies developing on yeast extract agar. It is rarely encountered.

b) The typical appearance in direct light of colonies containing only phase II antigen is identical with the R form described under *1c*. The two forms may, however, be differentiated in oblique light, as this type of illumination reveals that colonies falling into subgroup 2b contain no brown star formation. At plate position 2–4 the whitish, opaque colonies belonging to the latter subgroup may contain some small, indistinctly limited yellowish or brownish spots, but no sharply limited stars. Sometimes S-like colonies also lack the brown star formation; these are classified in this same subgroup. These colony forms are agglutinable only in phase II serum. Their equivalence with colony type 2b grown on yeast extract agar can be confirmed by means of cross-subcultures.

c) These types of colony resemble the former ones, excepting that they are flatter and less opaque.

In freshly isolated cultures type 2b and 2c colonies were generally rare. As a very unfrequent occurrence the subculture from DC plates (used for primary isolation) consisted purely or chiefly of type 2b colonies.

Group 3. The *R* variants were regarded as members of this group. In direct light the colonies were pinhole or pinhead sized, yellowish-white, opaque, raised or low raised, circular, oval or slightly angular with a smooth, glistening surface and a sharp or sometimes finely crenated edge. In oblique light at plate position 4–8 a brownish, indistinctly limited area is visible in the centre, while the periphery of the colony is whitish, granular or distinctly reticular. Occasionally the netted structure consisting of white dotted lines extends over the whole colony. These forms correspond to the reticular colonies developing on yeast extract agar. They are more or less agglutinable in *Sh. sonnei* *R* serum and often also in phase I serum. They give a spontaneous agglutination in 3 per cent NaCl and are trypaflavine positive.

In addition to those described, under certain conditions some further colony forms occur, e.g. variants containing exclusively antigen S, but differing from type 2a in morphology and other respects. These special variants require further investigation.

It is clear that on D agar *Sh. sonnei* cultures show the same type of dissociation that can be observed on yeast extract agar. D agar is, however, preferable, as on this medium colonies with brown star formation are easily detected.

Identification of Sh. sonnei. It was shown [5] that the examination of the morphological properties of *Sh. sonnei* colonies might be used for the identification of this organism. Colonies with star formation produced by some other bacteria belonging to the family *Enterobacteriaceae* could generally be distinguished from those of *Sh. sonnei*. Differentiation was possible on the basis of other morphological properties and colonial agglutination. When extending the examinations it has, however, been found that the morphological character of yeast extract agar colonies is not always sufficient for the differentiation between *E. coli* and *Sh. sonnei*, as stars formed by *E. coli* are sometimes weakly refractive. Alternatively, *Sh. sonnei* may produce highly refractive stars that can be recognized even in reflected or natural light. The reliable identification of *Sh. sonnei* is further enhanced by the use of D agar. Therefore the culture to be investigated should be streaked on both yeast extract and D agar plates. After incubating for one day the isolated colonies should be examined as described elsewhere [5]. *Sh. sonnei* cultures show generally a very characteristic dissociation; after some training, identification presents no difficulties.

Sh. sonnei strains freshly isolated from faeces or maintained in the refrigerator on DORSET's medium produce colonies with star formation. These colonies are agglutinable in both phase I and phase II sera. In addition to

these forms, moderately refractive, homogeneous colonies agglutinable only in phase II serum can be found. On D agar the corresponding colony forms 1a, 1b, 1c and 2b develop.

Among freshly isolated *Sh. sonnei* strains the occurrence of phase II (type 2b) colonies in pure cultures is rare. The subcultures of strains stored for protracted periods or not under proper conditions may, however, consist entirely of phase II colonies.

Exceptionally the subculture of a freshly isolated *Sh. sonnei* strain may contain exclusively highly refractive colonies on yeast extract agar, or may consist of the corresponding subgroup 2a colonies on D agar. Both cultures are agglutinable only in *Sh. sonnei* S serum.

Other *Shigella* types do not produce stars on either media. On D agar they form pinhole or pinhead sized colonies which are circular or spindle-shaped in direct light. No *R* forms appear. In oblique light at plate position 4–8 they are whitish and opaque. They can be mistaken only for *Sh. sonnei* colonies belonging to subgroup 2a. In this case slide agglutination may be used for differentiation.

E. coli strains producing stars on yeast extract agar grow in red colonies on D agar. The red colonies may appear in two different forms. One is convex, circular with a sharp edge and a smooth, glistening surface; the other is flat, more or less circular with a slightly crenated edge and raised margin, central papilla and granular surface.

No star formation was observed in colonies of bacteria belonging to the *Alkalescens*–*Dispar* group.

By the method described 441 strains were examined (116 *Sh. sonnei* and 325 other enteric bacteria). The method was found adequate for the simple quick and reliable identification of *Sh. sonnei*.

Discussion

The dissociation forms of *Sh. sonnei* were differentiated first on the basis of colonial morphology [2]. Subsequently the difference between the antigenic structure of these forms was revealed [4]. Between the morphology and antigenic structure, however, no such close connection was demonstrated, which, on the basis of colonial appearance, would have provided reliable information about the antigenic contents of different strains [1, 3]. The problem of the dissociation of *Sh. sonnei* requires further investigation. The elucidation of some questions has been delayed by the fact that various antigenic phases may occur in similar colony forms, or, on the other hand, in one and the same colony various phases may simultaneously be present. Adequate phase determination of various colonies of a *Sh. sonnei* strain was therefore not possible

either by morphological or serological examination. A procedure has been recommended to eliminate these difficulties. The new method can be applied both in research and in the routine identification of *Sh. sonnei*.

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Address of the author: BÉLA SERÉNY
State Institute of Hygiene, Budapest IX. Gyáli út 2/6.

ELECTRON MICROSCOPIC EXAMINATION OF COMPLETE AND INCOMPLETE INFLUENZA VIRUSES

III. EXAMINATION OF THE FINE STRUCTURE OF COMPLETE INFLUENZA VIRUS BY DISINTEGRATION AND DIGESTION METHODS

By

I. HOLLÓS

State Institute of Hygiene, Budapest

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Summary. Purified complete influenza virus mounted on formvar film was disintegrated and digested by a combined method. On trypsin digestion the well-known ring-like structure appeared. This disintegrated on combined treatment with ribonuclease and trypsin. On trypsin digestion after ether treatment filamentous formations were present beside small globules. The filaments disintegrated on combined ribonuclease and trypsin treatment. Suspended virus particles treated with ether yielded small spheroids and some short rods.

In a previous report we described a hypothesis concerning the fine structure of complete influenza virus revealed by some morphological studies in ultrathin sections [1]. The virus particles seemed to be surrounded by a structureless, homogeneous substance, inside of which the presence of an outer lamella covering the elementary body was demonstrated. The inner lamella and the adjacent electron-dense structural elements were observable only in certain particles cut at the appropriate level. The inner lamella was supposed to represent a part of a hemispheric structure, while the electron-dense elements appeared to be parts of an undulant filament at the margin of the above hemisphere. On the electron micrographs by MORGAN *et al.* [2] a structure very similar to that supposed by us is apparent. VALENTINE and ISAACS [3a, b] described a trypsin resistant ring inside the influenza virus, while HOYLE *et al.* [4a, b, c] have found the virus to consist mostly of globules 120 Å in diameter, incorporated in an ether soluble lipid material. The Rostock strain of the classic fowl-plague virus when desintegrated by ether was found by SCHÄFER and ZILLIG [5] to consist of RNA containing soluble-antigen globules, having a great inclination to arrange themselves in chains.

The purpose of the present study was to obtain further data on the fine structure of complete influenza virus, using the methods quoted above.

Materials and methods

As to the virus strain and the characterization of the virus material we refer to one of earlier reports [1].

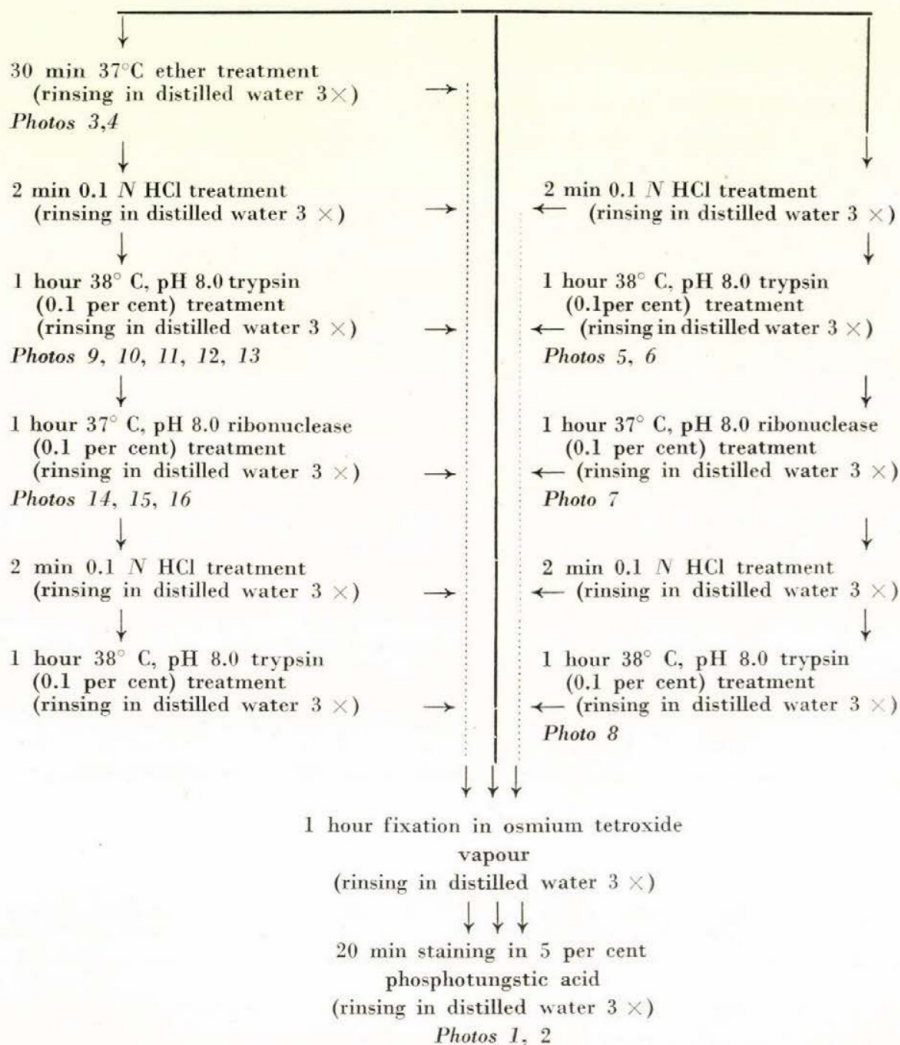
Preparation for electron microscopy. Drops of purified complete influenza virus suspension in saline were carried onto microscreens provided by formvar microfilms. After 1 minute the preparates were washed by immersion into 3 successive baths of distilled water

using fresh distilled water for each series of preparates. This procedure always preceded the single steps of preparation independently of the actual method used. Samples were taken from the preparates for fixation and examination at each phase of their preparation.

For fixation treatment by OsO_4 vapour (1 per cent solution) for 1 hour was followed by washing in distilled water as described above, then treatment with a buffered 5 per cent solution of phosphor-tungstic acid in a moist chamber, followed again by washing in distilled water. The preparate was then allowed to dry.

The scheme of our preparation procedure is presented in Table I. Care was taken to avoid drying of the preparates during the procedure.

Table I
Complete influenza virus



Half of the complete influenza virus preparates described above was subjected to 30 minutes' ether treatment using a constantly readjusted 2 cm overly of boiling ether in a

water bath of 37° C. At the 15th minute of the treatment the total amount of ether was replaced by a fresh one.

After ether treatment both the treated and the untreated preparates were handled in a similar way as described below. After 2 minutes' pretreatment by 0.1 *N* hydrochloric acid the preparates were digested for 1 hour at 38° C in 0.1 per cent trypsin in phosphate buffer of pH 8.0. The trypsin solution was replaced by a fresh one after 30 minutes.

The next step of preparation was treatment by 0.1 per cent ribonuclease in phosphate buffer of pH 8.0 at 37° C for one hour. Similarly to the trypsin solution, the ribonuclease was also replaced by a fresh solution at the 30th minute of treatment.

Digestion by ribonuclease was followed by additional treatment with 0.1 *N* HCl and trypsin as described above.

Complete influenza virus was subjected to ether disintegration also in suspension. Equal amounts of ether and purified influenza virus suspension were mixed and allowed to stand at 37° C for an hour in a hermetically closed flask. The mixture was shaken from time to time and after 1 hour it was centrifuged at 3000 RPM for 10 minutes. The ether residue was evaporated from the watery phase and the ether treated suspension was carried onto formvar film, fixed and dried as described above.

Preparations were examined either unshadowed, palladium or gold shadowed, using an EM 3 USSR 1951 electron microscope readjusted by HOLLÓS and BARNA. Micrographs were taken onto "Agfa Dokument" film, with an electronoptical magnification of about 6 000 to 18 000 times.

Results

Photomicrographs 1 and 2 present complete influenza viruses. Within the unshadowed elementary bodies there are the dim outlines of some structure. The shadowed particles are practically identical and display the usual uneven surface.

Photomicrographs 3 and 4. The elementary bodies subjected to mild treatment with ether, though not regularly spherical, still do not seem to have disintegrated to small granules. No remarkable change was caused by treatment with 0.1 *N* hydrochloric acid for 2 minutes in either the ether treated or the untreated particles while on treatment with enzyme striking differences were apparent between the ether pretreated and untreated preparations.

The statements by VALENTINE and ISAACS were found to be valid also for the Paris strain used by us. Without previous ethyl-ether treatment after trypsin digestion (*Photomicrographs 5 and 6*) the appearance of the ring-like structure was observed. The rings were either empty or they contained some material of very low electron density. Within the ring at certain parts some more dense lines were faintly seen. In some of the rings a small intumescence pointing towards the centre of the particle was found. The trypsin resistant rings were greater in diameter than the original elementary bodies.

No remarkable changes occurred in the typical ring-like structure on treatment with ribonuclease (*Photomicrograph 7*) and 0.1 *N* hydrochloric acid. A second treatment with trypsin (*Photomicrograph 8*) caused the ring-like structure to disintegrate into irregular particles, essentially similar in both the ether-pretreated and the untreated preparations.

Mild pretreatment with ether on the formvar film and a single digestion by trypsin induced morphological characteristics different from those mention-

ed above. In such preparations most of the typical trypsin resistant rings had been replaced by globules and filaments. (*Photomicrographs 9 to 13.*) The filaments were usually structureless except those crossing each other; these contained a faint internal structure. The particles on *Photomicrograph 11* have moderately disintegrated and some ring-like residues are well recognizable. In the unshadowed preparation shown in *Photomicrograph 13* the sub-units of the filaments differ in electron density.

Ribonuclease treatment (*Photomicrographs 14 to 16*) left the globules intact, while the filaments became fainter, less structured and some of them thinner. Beside the single filaments, double and crossed ones were often found. The length of the individual filaments exhibited a wide variation.

After a second treatment with 0.1 N hydrochloric acid and trypsin only some totally disintegrated material was left, but no filaments. The moderate number of ring-like structures still observable in some preparations appeared to be of cellular rather than viral origin.

As we were unable to demonstrate the disintegration of virus mounted on formvar to small spherical bodies on the effect of ether treatment, the experiment was repeated with suspended virus particles. *Photomicrograph 17* presents the typical spherical bodies obtained on ether treatment of a virus suspension. Some of these bodies assumed chain-like or aggregate forms; straight or curved, structureless rods mixed with the globules were also observed.

Discussion

Enzymatic digestion of virus preparations may yield a wide variety of results if previously the particles have not been made sensitive to the enzyme actually used.

Plate 1 (Photomicrographs 1 to 9)

Magnifications about 50 000 times.

Photomicrograph 1. (No. 2 233) Complete influenza virus. In the particles designated by an arrow a complex structure composed of different details can be seen. Unshadowed.

Photomicrograph 2. (No. 17 107) Complete influenza virus. Gold shadowed.

Photomicrograph 3. (No. 14 679) Formvar film mounted complete influenza virus after 30 min. ether treatment at 37° C. Contours of the elementary bodies unchanged, though a certain polymorphism is realizable. No formation of spheroids. Unshadowed.

Photomicrograph 4. (No. 17 429) Same as Photo 3. Damaged particles, but no formation of spheroids. Gold shadowed.

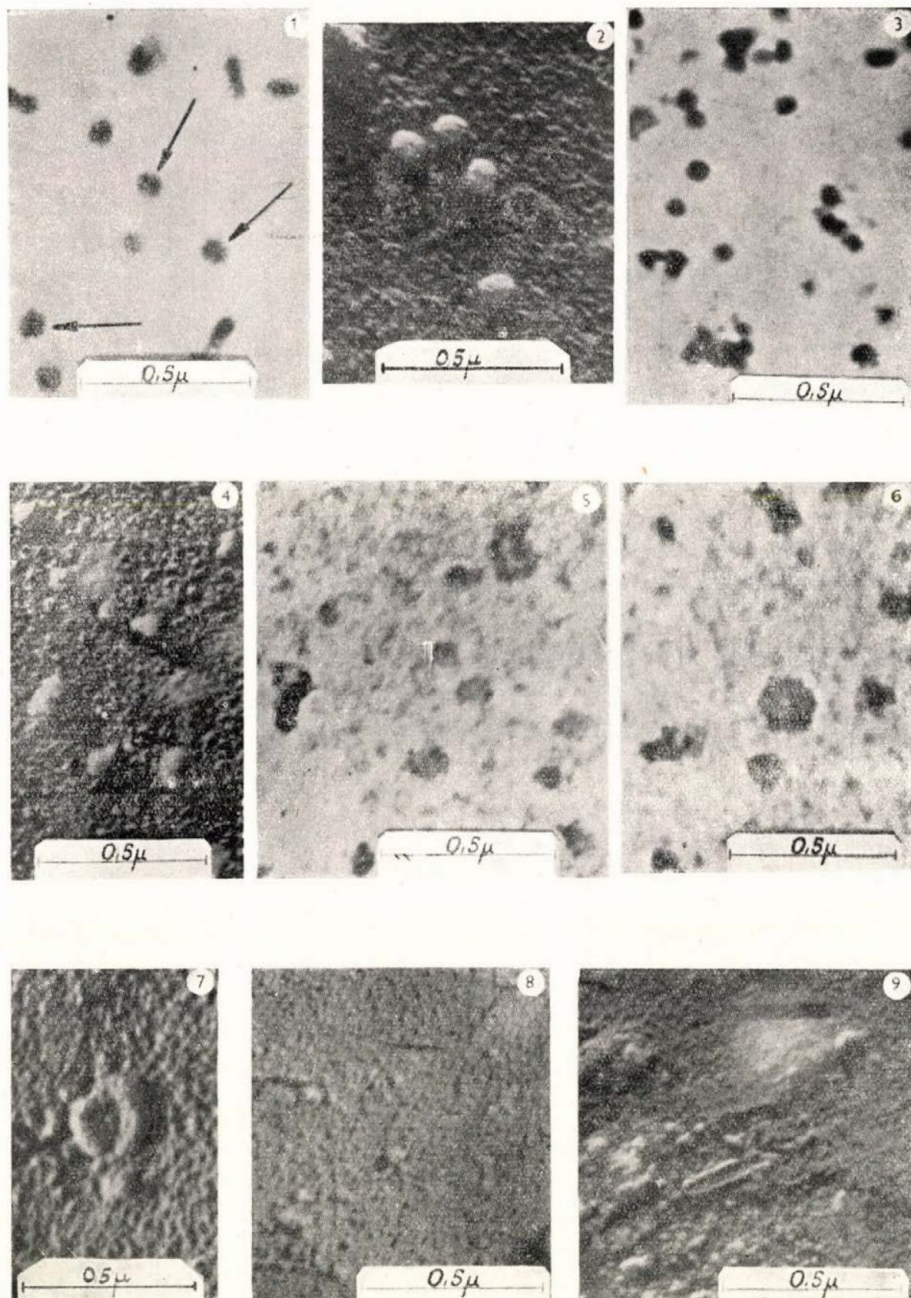
Photomicrograph 5. (No. 11 387) Complete influenza virus after trypsin treatment. Larger particles exhibit ring-like shape surrounding a less electrondense material. In the ring there are one or two lines of greater electron density. Unshadowed.

Photomicrograph 6. (No. 11 388) Same as photo 5.

Photomicrograph 7. (No. 12 889) Virus particle treated with trypsin and ribonuclease. Morphology essentially similar to that on Photos 5 and 6. Gold shadowed.

Photomicrograph 8. (No. 17 923) Particles obtained after successive treatment with trypsin-ribonuclease-trypsin. Irregular small particles. Palladium shadowed.

Photomicrograph 9. (No. 17 973) Particles treated with ether and trypsin after being mounted on formvar film. Among many globules some filamentous formations with no internal structure. Palladium shadowed.



Sensitization may be achieved by the use of certain fixatives [7] or by treatment with acid [3a]. Without pretreatment with acid influenza virus was found to be trypsin-resistant even if the enzyme was dissolved in phosphate buffer. The aspecific swelling effect of phosphate buffer [8] was not observed in our preliminary experiments, nevertheless there was no definite evidence to deny its possible influence on the diameter of the trypsin-resistant ring.

The outer protective lamella of complete influenza virus disintegrated on treatment with trypsin or ether. On digestion by trypsin the lamella lost its normal appearance and only a ring of trypsin-resistant material was left. The morphology of complete virus particles mounted on microfilm was left unchanged by mild ether treatment, while intensive treatment resulted in total disintegration of the particles. These two facts suggested the supposition that the lamella may contain both proteins and lipids. The internal structure of the trypsin resistant ring and its exact place inside the particle have not as yet been well defined. The fact that combined ribonuclease and trypsin treatment caused disintegration of the ring suggested the ribonucleoprotein character of this structural element. The formation of globules and long filaments on treating the particles with ether and trypsin and the disintegration of these structures on ribonuclease and trypsin treatment made the presence of structural lipoprotein highly probable. This view was further supported by the finding that rough ether treatment of particles in suspension resulted beside globule formation also in the appearance of rod-like fragments, most probably parts of the long filaments obtained by other methods.

As to the place of the trypsin resistant ring inside the particle we suggested a theory in our previous publication. We supposed it to represent an

Plate 2 (Photomicrographs 10 to 17)

Magnifications about 50 000 times unless otherwise stated.

Photomicrograph 10. (No. 14 951) Pretreatment as in Photo 9. Right and downwards from the amorphous material in the centre there are filamentous formations. Their majority is composed of small globules, while the long filament at the left contains crossed, wavy smaller filaments. Gold shadowed.

Photomicrograph 11. (No. 14 948) Same as Photo 10. The disintegration of the elementary bodies is less marked. The particle at the left exhibits residues of a typical ring. Gold shadowed. Magnification about 25 000 times.

Photomicrograph 12. (No. 18 000). Same as Photos 9–10–11. Palladium shadowed.

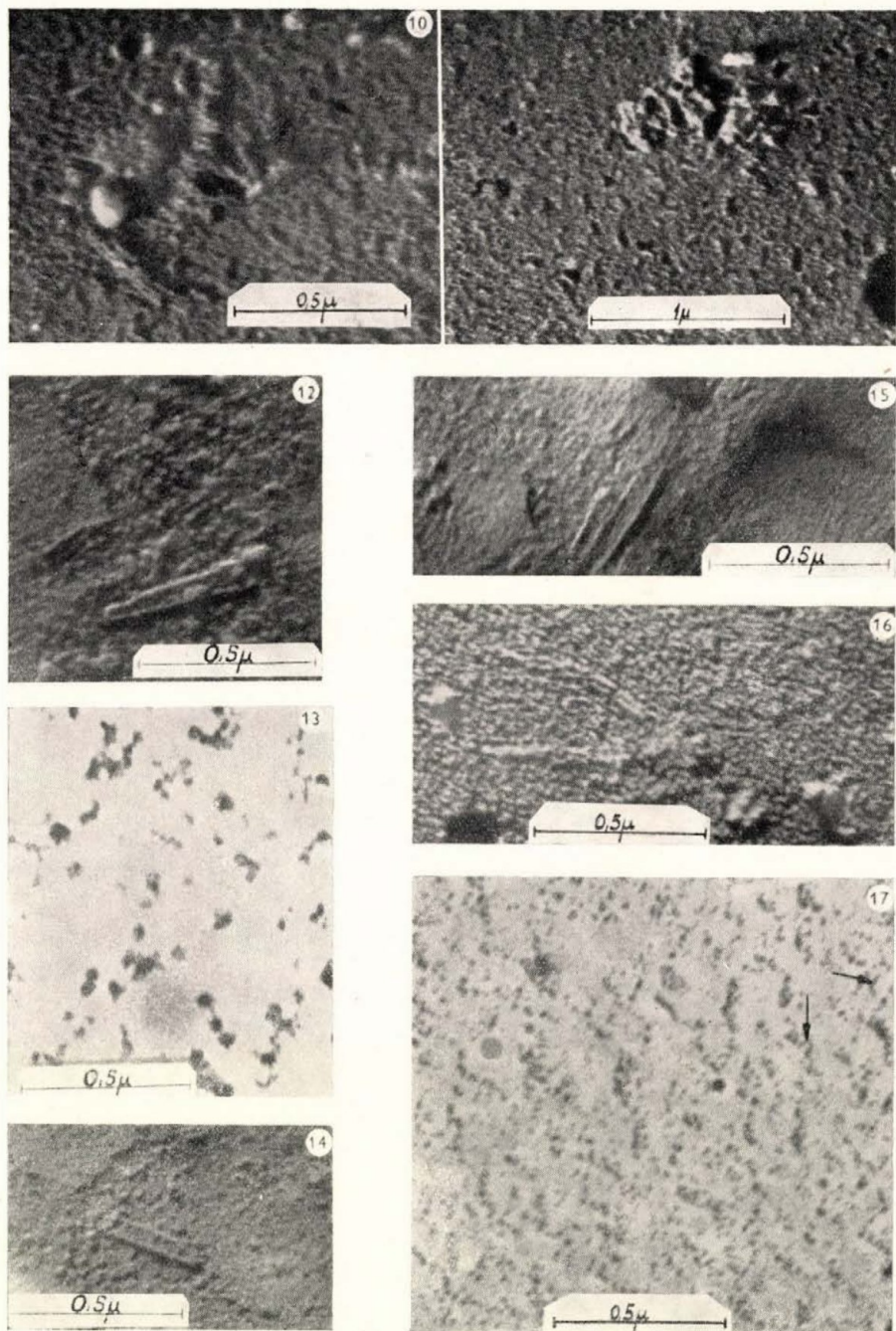
Photomicrograph 13. (No. 14 797) See photos on the previous picture. The subunits of the filaments differ in electron density. Unshadowed.

Photomicrograph 14. (No. 17 986) Formvar mounted particles after combined treatment with ether, trypsin and ribonuclease. Beside the globules two parallel filaments of different length are seen. The filaments seem to be finer than those not treated with ribonuclease. Palladium shadowed.

Photomicrograph 15. (No. 18 005) Same as Photo 14. Palladium shadowed.

Photomicrograph 16. (No. 15 024) Same as Photos 14, 15. Gold shadowed.

Photomicrograph 17. (No. 17 317) Particles treated in suspension with ether. There are typical spherical bodies and straight or curved, short structureless rods. Unshadowed.



empty hemisphere carrying a ribonucleic acid or ribonucleoprotein filament along its wavy margin.*

The elementary body penetrating into the cell is exposed to the action of both proteolytic and lipolytic enzymes. As a result of digestion by these enzymes the typical appearance of the virus is supposed to disintegrate and the replicable ribonucleoprotein filaments escape. The question remains open whether or not the filaments disintegrate to smaller subunits inside the cell.**

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Address of the author: IVÁN HOLLÓS,
State Institute of Hygiene, Budapest IX. Gyáli út 2/6.

* After this work had been completed has appeared the report of A. BIRCH-ANDERSON and K. PAUCKER (*Virology*, **3**, 21, 1959) dealing with studies on ultrathin sections. These observations do not support our hemisphere theory.

** After completion of the present study was the report by PAUCKER, K., BIRCH-ANDERSON, A. and VON MAGNUS, P., published in *Virology* **3**, 1 (1959). These authors identified the globules obtained by ether treatment of suspended virus particles as the haemagglutinin, while the short rods as S antigen. The mild combined ether and trypsin treatment of formvar mounted particles yielded filaments longer than theirs and exhibiting frequently a double or crossed pattern. The filaments disintegrated on combined ribonuclease and trypsin treatment. The rods representing the S antigen and demonstrated also in our laboratory are very probably fragments of the filaments obtained by mild ether and trypsin treatment.

RESULTS OF ACTIVE IMMUNIZATION OF CIVILIAN POPULATION AGAINST TETANUS

By

A. PETRILLA

State Institute of Hygiene, Budapest

(Received September 28, 1959)

Summary. In Hungary vaccination against tetanus was made compulsory in 1953. Since then children have been vaccinated in the second half year of life, and at 6 years of age, with diphtheria-pertussis-tetanus combined vaccine, finally, at 12 years of age with typhoid-tetanus combined vaccine. At each instance two inoculations are given at an interval of four weeks, and basal immunization is followed by a booster dose one year later. In 1958 the age group under 20 years might be regarded as vaccinated. For the vaccinated age group the tetanus morbidity rate, which amounted to 9.2 per 100 000 before the introduction of vaccination (average for 1951 and 1952), has decreased by 80 per cent to 1.7 per 100 000 (rate for 1958). Thus, in spite of the fact that from 5 to 10 per cent of the children have escaped compulsory vaccination, the results are satisfactory. The decrease in the incidence of neonatal (under one year of age) tetanus is attributable to other factors, *e. g.* to the substantial rise in institutional confinements. Another possible factor is that certain mothers, chiefly the younger ones, have been immunized. We failed exactly to establish the vaccination status of the tetanus patients. Eighty-five per cent of the injuries leading to tetanus were too mild to be shown to a doctor. In such cases active immunization is the only means of preventing tetanus.

Several authors [1, 2, 3, *etc.*] have reported on the protective effect of active immunization against tetanus in battle casualties of World War II. It has been stated that the incidence of tetanus in immunized persons was negligible as compared with the great loss caused by the disease during World War I. Regarding civilian populations, however, no epidemiological data on large-scale active immunization are available.

In Hungary, after having obtained favourable results in small areas, active immunization against tetanus was made compulsory in 1953. In the autumn of 1953, in the vaccination programme diphtheria toxoid was replaced by a combined diphtheria-pertussis-tetanus vaccine (*di-pe-te*). Infants from 6 to 11 months of age were given two injections at an interval of four weeks. In the same year 12-year-old children were given two doses of tetanus toxoid at the same time interval.

Since 1954 the schedule of compulsory vaccination against tetanus is as follows.

- Di-pe-te, 2 inoculations at 6–11 months of age.
- Di-pe-te, 1 inoculation at 18 to 23 months of age.
- Di-pe-te, 2 inoculations at 6 years of age.
- Single tetanus, 1 inoculation at 7 years of age.
- Typhoid-tetanus 2 inoculations at 12 years of age.
- Typhoid-tetanus 1 injection at 13 years of age.

Table I
Incidence of tetanus by age in Hungary 1951-1958

Age, year	1951	1952	1953	1954	1955	1956	1957	1958
Under 1 year	65	55	35	41	33	21	25	14
1	3	6	1	1	—	—	1	—
2	9	7	4	1	1	2	1	2
3	11	8	9	10	—	1	2	1
4	7	10	12	5	6	2	1	1
5	5	19	8	5	10	8	5	2
6	23	19	13	8	15	15	11	3
7	10	13	12	12	9	12	3	6
8	19	24	10	15	8	4	7	1
9	21	13	27	15	14	8	2	4
10	12	13	13	19	9	7	7	3
11	17	9	16	11	10	8	3	6
12	9	15	13	3	9	9	2	3
13	13	12	6	5	9	4	4	2
14	17	11	14	4	7	6	—	1
15	8	11	10	5	7	3	2	1
16	7	4	11	2	5	2	1	—
17	16	13	9	3	6	—	2	3
18	11	3	8	2	—	4	1	1
19	4	7	3	5	—	4	4	—
20-29	39	46	46	28	18	20	17	27
30-39	46	43	42	30	39	27	22	29
40-49	59	60	62	45	47	48	32	32
50-59	39	54	49	54	47	48	50	49
60-69	50	38	34	38	38	20	30	29
70 and over	30	33	27	28	24	39	22	33
Unknown	—	—	—	1	—	—	2	1
Totals	544	548	494	346	371	322	258	254

It is noteworthy that 5 to 10 per cent of the children escaped vaccination.

The schedule will be modified in 1960, when children already vaccinated against tetanus will complete the age of six resp. twelve years. From that time only on children 6 and 12 years of age will be re-vaccinated by one dose.

Some adult groups were also vaccinated against tetanus, such as the young military, injured subjects (also passively immunized), sportsmen, persons handling animals, traffic employees. In addition, in certain areas, with a high incidence of typhoid fever vaccination of the whole population against typhoid fever was made compulsory; in these cases combined typhoid-tetanus vaccine was used. In 1958, *e. g.*, in addition to the every year programme nearly 1 million persons were vaccinated with typhoid-tetanus vaccine. Unfortunately, the vaccination status of adult individuals cannot be established exactly. ERDŐS *et al.* [4] in 1958 demonstrated, as a result of the use of typhoid-tetanus vaccine some immunity to tetanus in nearly one third of the adult population.

In the present work we attempted to estimate the protective effect of vaccination.

In Table I age distribution of the tetanus cases of the last 8 years are presented. Figures in the separate areas represent the data of the age groups expectedly influenced by vaccination. It is to be noted, however, that vaccinated and non-vaccinated age groups cannot be separated definitely. Vaccination in the second half year of life has no influence upon the morbidity of infants under one year, as nearly all the cases under one year are neonatal

Table II
Incidence of tetanus grouped by age

Age, year	Case incidence		Morbidity per 100 000		1958 morbidity index if 1951-52 = 100
	1951-52 average	1958	1951-52	1958	
0-4	91	18	10.7	1.9	18
5-9	83	16	10.9	1.9	17
10-14	64	15	8.7	2.0	23
15-19	39	5	5.2	0.7	14
20-29	43	27	2.7	1.9	71
30-39	45	29	3.7	2.1	57
40-49	60	32	4.4	2.3	52
50-59	46	49	4.4	4.2	96
60	76	62	6.8	4.8	71
Totals	546	253	5.8	2.6	45

ones. Year by year more age groups, by 1958 each under 19 years of age, had received their basal vaccination. *Table I* shows that in the same age groups the incidence of tetanus in the vaccinated subjects was generally lower than in the non-vaccinated ones, and the incidence decreased after vaccination.

In *Table II* and *Fig. 1* the age distribution of cases for the period immediately preceding vaccination (1951–1952) is compared with that for 1958. As mentioned, for 1958, the great majority of the age group from 0 to 19 years may be regarded as having received at least 3 inoculations. The morbidity rate in this age group, which was 9.2 per 100 000 in the average of the years 1951 and 1952, had decreased by 80 per cent to 1.7 per 100 000 in 1958. Considering that the decrease was unfavourably influenced by those who escaped

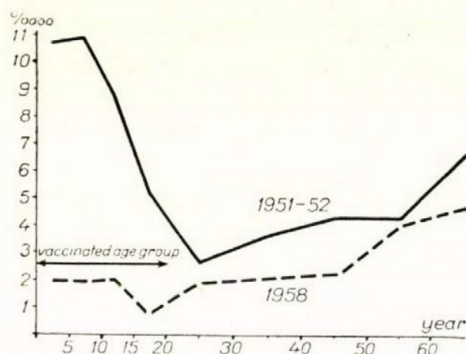


Fig. 1. Tetanus morbidity by age in Hungary

compulsory vaccination, the effect of vaccination may be regarded as satisfactory. A slight decline occurred in the incidence among persons over 20 years of age. This may be attributed to the occasional vaccinations on the one hand, and to various factors, such as more extensive medical care, wearing of shoes, and more general wearing of working-clothes, on the other.

It may be concluded that vaccination in the civilian population of Hungary against tetanus has been successful; the incidence of the disease has been reduced by 80 per cent.

The decrease in the incidence of neonatal tetanus was remarkable. The average case-incidence for the years 1951 and 1952 was 60, with nearly every case fatal in outcome. In contrast, as few as 14 cases were reported in 1958. As shown by *Table I*, the incidence of tetanus among infants under one year of age has been decreasing from year to year. This might be explained by the substantial rise in hospital deliveries. Institutional confinements made up 34.3 per cent of the total in 1950; 41.4 per cent in 1951; 69.2 per cent in 1957; 77.0 per cent in 1958. The rise was steeper in the villages where the corresponding ratios were 17.3, 23.5, 59.1 and 71.2 per cent. Maternal immunity might be another factor lowering the incidence of neonatal tetanus. According to

the 1957 figures (those for 1958 are not yet available) in Hungary 12 per cent of the live births fall to mothers aged from 15 to 19 years, thus belonging in the vaccinated age group. A substantial but undeterminable proportion of the older mothers must have also been vaccinated.

We have attempted, but with little success, to establish the vaccination status of the tetanus patients.

Table III

Distribution by vaccination status and outcome of illness of tetanus cases belonging in the age group involved in compulsory vaccination

Age year	Vaccinated		Vaccination status unknown		Non vaccinated		Total
	recovered	died	recovered	died	recovered	died	
1-2	—	1	1	—	—	—	2
3-5	—	—	1	—	2	1	4
6-9	6	1	4	2	1	1	15
10-14	4	4	1	—	2	4	15
Totals ...	10	6	7	2	5	6	36

Table III shows the vaccination status for some patients belonging in the vaccinated age group. In the age group from 1 to 14 years a total of 36 cases occurred in 1958. Sixteen patients had been vaccinated; in 9 cases the vaccination status could not be established while 11 patients were reported as non-vaccinated. These data, being based on the memory of the relatives, are not quite dependable. It is nevertheless of some interest that round 30 per cent of the patients was reported as non-vaccinated. It has been mentioned, that as few as 5 to 10 per cent of the children escaped compulsory vaccination.

Table IV

Distribution of tetanus cases by case history

Case history	Cases in	
	1952	1958
Operation or some other medical intervention	11	3
Neonatal case	46	14
Injury seen by doctor	55	30
Injury not seen by doctor	362	194
Unknown	74	13
Totals	474	241

In *Table IV* patients are grouped on the grounds whether they were seen by a physician after they had suffered some injury. Postoperative and neonatal cases, and cases with an uncertain history have been omitted. Of the rest we succeeded in establishing the data of 417 patients for 1952 and of 224 for 1958. *Accordingly, 85 and 84 per cent, respectively, of the patients had not been seen by a doctor and thus had not been immunized passively.* It has been concluded that tetanus may be prevented by passive immunization, but minor injuries causing the great majority of the tetanus cases, are unattainable for passive immunization. In such cases tetanus may be only prevented by systematically and repeatedly administered active immunization.

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Address of the author: ALADÁR PETRILLA,
State Institute of Hygiene, Budapest IX., Gyáli út 2/6.

ÜBER DIE HERSTELLUNG UND ANTIGENEIGENSCHAFTEN VON CHROMOVALBUMIN

Von

L. KESZTYŰS, MARIA KÁVAY, E. SZABÓ und L. KOCSÁR

Pathophysiologisches Institut der Medizinischen Universität, Debrecen

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Zusammenfassung. 1. Es wurde ein Verfahren ausgearbeitet, nach dem natives Ovalbumin befriedigend chromiert werden kann.

2. Nach den Resultaten der Anaphylaxieversuche und der Ringpräzipitation verhalten sich das hergestellte Chromovalbumin und das native Ovalbumin als homologe Antigene.

3. Die Agarpräzipitation ergab, daß das native Ovalbumin drei Antigenfraktionen enthält; dementsprechend können auch im Immuserum drei Antikörper nachgewiesen werden. Chromovalbumin erscheint, wenn es mit Chromovalbumin-Immuserum reagiert, immunologisch einheitlich, und auf dieser Grundlage wäre anzunehmen, daß der Chromierungsprozeß die drei Antigenfraktionen des nativen Ovalbumins uniformiert. Da indessen Chromovalbumin, wenn es mit nativem Ovalbumin-Immuserum reagiert, die Anwesenheit von drei verschiedenen Antigenen anzeigt, dürfte als wahrscheinlicher zu betrachten sein, daß diese scheinbare Uniformierung auf Verschiebungen in der relativen Konzentration der Antigen-Antikörperpaare beruht.

In der Immunochemie benutzt man zur Markierung von Eiweiß im allgemeinen Isotopjod, Schwefel, Phosphor, Kohle, ferner Brom und die Nitrogruppe [1, 2, 3]. Alle diese zählen zu den Bioelementen, deren Stoffwechsel, Speicherung und Ausscheidung im Organismus auf mehr oder minder spezifische Weise vor sich geht. Unsere Kenntnisse über das Schicksal der Antigene stammen vorwiegend aus Versuchen, die mit Verbindungen durchgeführt wurden, welche derartige markierte Bioelemente enthielten. Interessanterweise hat man bisher selten Versuche mit Antigenen vorgenommen, die mit Elementen markiert waren, welche für den Organismus fremd sind, darin physiologisch nicht vorkommen. Nachdem unlängst bekannt wurde, daß man Cr^{51} zur Markierung von Erythrozyten [4] sowie des Coli-Endotoxins [5] mit guten Resultaten benutzt hat, schien es angezeigt, die neue Markierungstechnik auch bei Versuchen mit Eiweißantigenen zu erproben.

Die Halbwertszeit von Cr^{51} beträgt 26,5 Tage; das ist wesentlich vorteilhafter als die 8tägige Halbwertszeit von J^{131} . Die Verbindung ist leicht zu beschaffen und daher für kontinuierliche Versuche geeigneter als Jod.

Bevor wir indessen die Versuche begannen, schien es unbedingt nötig zu klären, welchen Einfluß der Chromierungsprozeß auf die Spezifität eines bekannten Antigens ausübt. In letzter Zeit haben wir mit Ovalbumin ziemlich viele Erfahrungen gesammelt, weshalb wir zur Untersuchung dieser Frage die Chromierung von Ovalbumin wählten. Naturgemäß verwendeten wir bei diesen Versuchen nicht Cr^{51} , sondern natürliches Chrom.

Methodik

Chromovalbumin versuchten wir zuerst nach dem von BRAUDE, CAREY, SUTHERLAND und ZALESKY [5] für Endotoxin ausgearbeiteten Verfahren herzustellen, und zwar unter Berücksichtigung der Differenz zwischen dem Molgewicht von Ovalbumin und Endotoxin. 100 mg Ovalbumin wurden mit der vorgeschriebenen Menge CrCl_3 bzw. Na_2CrO_4 in Phosphatpuffer von pH 7 bei 37°C 24 Stunden inkubiert und bei Zimmertemperatur mit dest. Wasser solange dialysiert, bis die Cr-Probe in der äußeren Flüssigkeit negativ ausfiel. Schon im Laufe der Inkubation kam die Fällung in Gang, die während der Dialyse außerordentlich stark wurde, so daß im dialysierten Produkt Chrom nur noch in Spuren nachzuweisen war. Die für Coli-Endotoxin ausgearbeitete Methode hat sich demnach zur Chromierung von Ovalbumin als ungeeignet erwiesen.

Auf Grund der bei Jodierung von Ovalbumin gewonnenen Erfahrungen gingen wir schließlich folgendermaßen vor:

Nach WARNER und WEBER [6] hergestelltes, dreimal umkristallisiertes Ovalbumin wurde in dest. Wasser gelöst, $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ zugegeben, die gewonnene Lösung 24 Stunden bei 37°C inkubiert und dest. Wasser gegenüber 48 Stunden lang bei $4-5^\circ\text{C}$ dialysiert, bis in der äußeren Flüssigkeit Chrom nicht mehr nachzuweisen war.

Hiernach bestimmten wir den N- und Cr-Gehalt des Chromovalbumins nach SCHULEK [7], mit Mikro-Kjeldahl bzw. nach dem kolorimetrischen Verfahren von ROWLAND [8]. Bei letzterem modifizierten wir die Destruktionstechnik und verbanden sie mit der später erforderlichen Oxydation.

Tabelle I zeigt den Cr-Gehalt des Ovalbumins in Abhängigkeit von der zugegebenen Chrommenge.

Tabelle I

Nr.	Zu 1,8 g Ovalbumin gegebene Cr^{+++} - Menge, mg	Cr-Gehalt des chromierten Ovalbumins	
		in %	Atome/Ovalb.- Mol.
1.	401,1	Das Eiweiß wurde aus- gefällt	
2.	199,2*	1,45	12,00
3.	100,3	1,35	11,79
4.	100,3	1,33	11,48
5.	100,0	1,34	11,56
6.	49,8	1,05	9,02
7.	24,9	0,51	4,43

* = schwache Opaleszenz.

Wie aus Tabelle I ersichtlich, erwiesen sich die quantitativen Verhältnisse von Nr. 3—5 als optimal, weil hier das Eiweiß nicht ausgefällt wird und auch der Cr-Gehalt dem Maximum nahesteht. Bei einem Ovalbumin-Molegewicht von 45 000 werden von jedem Molekül 12 Cr^{+++} -Atome gebunden. Bei Verringerung der zugeführten Cr-Menge verminderte sich auch das Bindungsverhältnis.

Bei der schonenden Jodierung von Ovalbumin bindet bekanntlich jedes Molekül maximal gleichfalls 12 Jodatome. Bei der schonenden Jodierung entstehen Monojodderivate laut Modellversuchen mit Tyrosin und Histidin [9]; dies stimmte mit der Tatsache überein, daß Ovalbumin je Molekül 5 Tyrosin- und 7 Histidineinheiten enthält [10, 11, 12].

Zur Klarstellung der Verhältnisse versuchten wir, das maximal chromierte Ovalbumin nach der Methode von KORNGOLD und Mitarbeitern [13] zu jodieren. Hierbei wurde Chromovalbumin denaturiert, und das zur Jodierung benutzte J^{131} gewannen wir in der äußeren

Dialysierungsflüssigkeit annähernd zurück. Hieraus darf geschlossen werden, dass sich Cr bei der Chromierung an jene Teile des Ovalbuminmoleküls bindet, die auch zur Bindung von J geeignet sind.

Ergebnisse und Besprechung

Mit je 1 mg des auf diese Weise hergestellten Chromovalbumins sowie des dreimal umkristallisierten nativen Ovalbumins sensibilisierten wir s. c. Meerschweinchen, bei denen nach 21 Tagen durch i. v. Einspritzung von 4 mg Chrom- bzw. nativem Ovalbumin Schock ausgelöst wurde. Die Ergebnisse sind in *Tabelle II* angeführt.

Tabelle II

Meerschweinchen Nr.	Sensibilisierendes Antigen 1 mg s. c.	Reinjiziertes Antigen 4 mg i. v.		Bemerkungen
		Cr-Ovalbumin	Natives Ovalbumin	
1.	Chromovalbumin	++++		nach 3' verendet
2.		++++		„ 5' „
3.		++++		„ 4' „
4.		++++		„ 5' „
5.			++++	„ 6' „
6.			++++	„ 6' „
7.			++++	„ 4' „
8.			++++	„ 3' „
9.			++	leichter Schock, am Leben geblieben
10.	Natives Ovalbumin	++++		nach 4' verendet
11.			++++	„ 4' „

Die Zahl der Kreuze (+) bezeichnet die Schwere der anaphylaktischen Symptome.

Von einem Versuch abgesehen, verhielten sich natives und Chromovalbumin als homologe Antigene und gaben Kreuzreaktionen. Die Chromierung war demnach, wie die Anaphylaxieversuche zeigten, ohne Einfluß auf die Antigenspezifität des Ovalbumins.

In weiteren Versuchen immunisierten wir jeweils 3 Kaninchen i. v. mit zunehmenden Mengen von nativem und Chromovalbumin. Die gewonnenen Immunsera wurden beiden Antigenen gegenüber mit der Ring- bzw. Agarpräzipitation untersucht.

Nach den Ergebnissen der Ringpräzipitation verhielten sich natives und Chromovalbumin als homologe Antigene, d. h. ihre Immunsera gaben Kreuzreaktionen von gleichem Ausmaß.

Die Agarpräzipitation wurde nach der Methode von JENNINGS [14] vorgenommen. Je ein mit Ovalbumin- bzw. Chromovalbumin-Immunserum gewonnenes Resultat veranschaulicht *Abb. 1*.

Wie aus *Abb. 1/a* hervorgeht, gibt natives Ovalbumin mit dem homologen Immunserum 3 gut nachweisbare Präzipitationsstreifen. Dies beweist, daß es 3 Antigenkomponenten enthält. Elektrophoretisch konnte Ovalbumin eben-

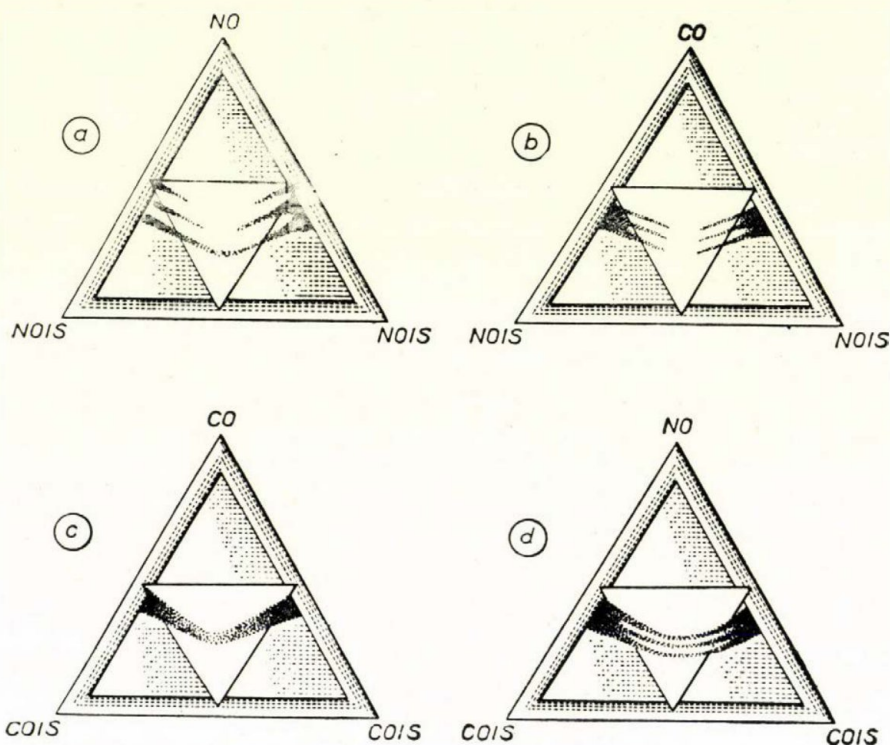


Abb. 1. NO = Natives Ovalbumin
 NOIS = Natives Ovalbumin-Immunserum
 CO = Chromovalbumin
 COIS = Chromovalbumin-Immunserum.

falls in 3 Komponenten getrennt werden [15]. Nach *Abb. 1/c* gibt Chromovalbumin mit Chromovalbumin-Immunserum einen einzigen dicken Präzipitationsstreifen, der im ersten Augenblick den Anschein erweckt, als ob Chromovalbumin immunologisch einheitlich und nicht in Fraktionen zu trennen sei.

Abb. 1/b jedoch zeigt, daß dieses homogen erscheinende Chromovalbumin mit dem nativen Ovalbumin-Immunserum ebenfalls 3 gut nachweisbare Streifen gibt.

In *Abb. 1/d* hingegen ist deutlich zu sehen, daß auch Chromovalbumin-Immunserum mit dem nativen Ovalbumin — den drei Antigenfraktionen

entsprechend — 3 Linien ergibt, obwohl es vorher Chromovalbumin gegenüber einheitlich erschienen war.

Die Agarpräzipitationsresultate zusammenfassend, dürfen wir feststellen, daß das native Ovalbumin in die Fraktionen A_1 , A_2 und A_3 getrennt werden kann und auch im nativen Ovalbumin-Immunserum drei Antikörper (Anti- A_1 , Anti- A_2 und Anti- A_3) nachzuweisen sind. Bei der Untersuchung von Chromovalbumin sahen wir, daß bei der Reaktion des nativen Ovalbumin-Immunserums und des Chromovalbumins drei Präzipitationsstreifen zustande kommen. Ebenso entstehen drei Präzipitationsstreifen, wenn Chromovalbumin-Immunserum mit nativem Ovalbumin reagiert. Auf Grund dieser Ergebnisse enthält die Chromovalbuminlösung drei verschiedene Antigene, da sich die je drei Präzipitationsstreifen sonst schwer erklären ließen. Nachdem in Systemkomplexen unter gewissen Umständen auch verwandte Antigene das Erscheinen besonderer Präzipitationsstreifen herbeiführen, läßt sich die Verwandtschaft der Antigene im Falle der je 3 Antigenssysteme natürlich nicht ausschließen. Die Tatsache, daß Chromovalbumin-Immunserum bei der Reaktion mit Chromovalbumin-Antigen zur Entstehung eines einzigen Präzipitationsstreifens führt, schließt die Voraussetzung der drei Antigen-Antikörpersysteme nicht aus, weil die Zahl der Präzipitationsstreifen die minimale Zahl der Antigen-Antikörpersysteme ausdrückt. Es läßt sich daher denken, daß bei der Reaktion von Chromovalbumin-Antiserum mit Chromovalbumin die relative Konzentration der drei Antigen-Antikörperpaare einander ähnlich war und dies dazu geführt hat, daß die Streifen der drei Systeme in den gleichen Streifen des Dreiecks entstanden, einander überdeckten und auf diese Weise ein einziger Streifen zustande kam. Ähnliche Erscheinungen wurden mehrmals in der Literatur mitgeteilt. Die Verringerung der Streifenzahl kann auch darauf beruhen, daß sich in Systemkomplexen die relative Konzentration der verwandten Antigene verändert, indem die relative Konzentration des über die meisten identischen Antigen determinanten verfügenden (»homologen«) Antigens höher wird als die der über weniger gleiche Antigen determinanten verfügenden (»heterologen«) Antigene.

Weniger wahrscheinlich ist die Annahme, daß sich die drei Antigenfraktionen des nativen Ovalbumins infolge der Chromierung uniformiert haben und deshalb auch das durch Immunisierung mit Chromovalbumin gewonnene Antiserum nur einen uniformierten Antikörper enthalten würde. Eine wesentliche Spezifitätsveränderung kann indessen auch bei der gegebenenfalls denkbaren Uniformierung von Chromovalbumin nicht eintreten, weil auch der uniformierte Antikörper des Chromovalbumin-Immunserums imstande ist, mit allen drei Ovalbumin-Fraktionen zu reagieren.

Auf Grund dieser Hypothesen wollen wir in weiteren Versuchen endgültig klären, ob es sich bei dem Chromovalbumin-Antigen- und Chromovalbumin-Immunserum-System wirklich um dreipaarige Systemkomplexe handelt oder

ob sie einem homogenen Antigen-Antikörpersystem entsprechen, in dem sich die drei Fraktionen des nativen Ovalbumins uniformiert haben, indem sie einen gemeinsamen Komplex bildeten.

Die bisher durchgeführten quantitativen Präzipitationsuntersuchungen haben jedoch den homologen Zustand des Chromovalbumins und nativen Ovalbumins nicht bestätigt. Wir gewannen unerwartete Ergebnisse, die unbedingt mehrmals reproduziert und von mehreren Seiten nachgeprüft werden müssen, bevor wir sie auswerten und veröffentlichen.

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Die Adresse der Autoren:

LÓRÁND KESZTYÜS, MÁRIA KÁVAY, ENDRE SZABÓ, LÁSZLÓ KOCSÁR,
Pathophysiologisches Institut der Medizinischen Universität, Debrecen, Ungarn.

STUDY OF A VACCINE STRAIN OF MYCOBACTERIUM TUBERCULOSIS

By

J. WEISSFEILER and VALENTINA KARASSOVA

State Institute of Hygiene, Budapest

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Summary. (i) Strain No 115 is a variant of human *Mycobacterium tuberculosis* with attenuated virulence.

(ii) The attenuated virulence of strain No 115 did not undergo any change during 4 years' passage in guinea pigs.

(iii) Inoculated into guinea pigs, the strain continued to multiply for 4 to 5 months, and could be obtained in cultures from the organs even as late as 236 days following inoculation.

(iv) Subcutaneous or oral vaccination of guinea pigs with strain No 115 was followed by an immunity of higher order than that brought about by BCG.

(v) Pathogenicity of strain No 115 in monkeys was found to be attenuated. On the basis of the above, strain No 115 may be considered a vaccinating strain.

Research and practical observations with the BCG strain evolved by CALMETTE and GUÉRIN have furnished evidence of the harmlessness and efficacy of this vaccine. Efficiency of vaccination against tuberculosis might, however, be significantly increased by the use of a strain with attenuated virulence and possessing immunogenic properties of a higher order. The BCG strain is of the bovine type, and the hypothesis that their immunogenic action is more pronounced to virulent bovine type than to virulent human strains, has been confirmed experimentally [5].

In 1940 WELLS and BROOK [13] pointed out that the murine type of tubercle bacillus the Vole bacillus, obtained from field-mice (*Microtus agrestis*) by WELLS, does not cause virulent infection in guinea pigs, and its inoculation produces more complete and more durable immunity than BCG does. But trials with the Vole strain did not yield more favourable results, either in animals or in man, and gave sometimes rise to serious complications; in England comparative studies on children showed that its immunizing power is not superior to that of BCG [1]. These observations agree with our own experimental results and the conclusions drawn from them [6].

GRÄUB's bovine vaccine strain has been studied by MANNINGER and KEMENES [2] in the calf. The animals were exposed to natural infection and revaccinated at six-month intervals. The authors verified the immunizing effect of this vaccine, but as they had no control group vaccinated with BCG, they were unable to draw comparative conclusions concerning the immunogenic action.

In the course of the extensive studies with the BCG strains certain requirements have been formulated to be fulfilled by living vaccines [3, 4, 5, 7]. These requirements are the following.

(i) The vaccine strain must be of attenuated virulence. This means that inoculated into the organism the strain should cause no malignant changes, but tissular and humoral reactions necessary for the development of immunity. This process is called the vaccination process; it is characterized by proliferation of the inoculated bacteria for a limited period of time, and regression of tissular changes within a certain period of time [3, 4].

(ii) The properties of the vaccine strain should be stable and hereditary. Its virulence must not increase, either during passage into the organism or under any other influence, because such an increase would imply the loss of one of its most essential properties — harmlessness. Diminution of virulence is also undesirable; this would involve a change in the capacity leading to the development of the vaccination process and immunity.

(iii) The vaccine strain should be of attenuated virulence not only in relation to the laboratory animals usually employed, but also to monkeys and naturally also in relation to man himself.

(iv) The vaccine strain must bring about immunity of a high order. Since BCG strains are widely employed, any new vaccine strain will assume importance only if its immunogenic properties are of a higher order than those of the BCG strain.

In the course of our studies of the variability of *Mycobacterium tuberculosis*, since 1933 we have been investigating the immunogenic properties of numerous avirulent and attenuated tuberculosis strains obtained by ourselves [5, 8, 9]. As a result, we have obtained a strain with properties fulfilling the above requirements. This strain, designated No 115, has been subjected to careful investigation; in the present paper we give a report on this work.

Materials and methods

Origin and properties of strain No 115. While studying the filterable forms of *M. tuberculosis* we found that upon adsorption on aluminium hydroxide gel and inoculation into guinea pig or white mouse, they were regenerated, and 30 to 45 days later acid-fast Mycobacteria could be cultivated from the animals [10]. In January, 1953, this method of adsorption-regeneration was employed for the demonstration and investigation of the filterable forms occurring in the blood of a woman with pulmonary tuberculosis. From a guinea pig (see Fig 1, No 103), inoculated with adsorbed haemolysed blood, we succeeded in cultivating the strain of attenuated virulence [11] which, having been obtained by passage through guinea pig No 115, was designated by that number.

This strain No 115 is a typical R-variant, forming uneven colonies on solid medium, and growing in the form of a rough, plicate membrane on fluid media. Proliferation is slow, on Löwenstein medium the colonies appear in 8 to 10 days.

Methods. — Investigations of strain No 115 were started in Tashkent in 1953, by passage in guinea pigs. The pathogenic properties of the strain were studied also in white mice, rabbits and monkeys. Its immunogenic capacity was investigated in guinea pigs. From the number of colonies produced by cultures of lymph nodes and organs within a certain period of time following inoculation, the rate of proliferation was estimated. Passage was

performed either directly with the organs of laboratory animals or with cultures grown from them.

The immunogenic power of the strain was tested by our method developed in 1935 [8] and elaborated further in 1944 [12], based on the following procedure. Ten to 15 animals are inoculated subcutaneously in the inguinal region with 0.01 mg of the strain to be investigated. A group comprising an equal number of animals is immunized simultaneously with similar doses of BCG culture. Oral immunization is carried out with three 10 mg doses of the culture, given on an empty stomach at two-day intervals. 30 to 45 days following immunization, immunized animals and 10 to 15 of the controls are infected with 0.00001 mg of virulent culture on the other side, subcutaneously. 3 to 4 months after infection, when the controls have developed generalized tuberculosis, all the animals are sacrificed; local infections, tuberculous and other changes in the lymph nodes and interior organs (lung, spleen, liver) are examined and subjected to histological investigation.

The gravity of tuberculous infection is expressed by crosses: changes in the regional lymph nodes and lymphatic system, the lung, spleen, liver, are each marked with maximum four crosses, changes at the site of infection with two. Thus in grave tuberculosis of all organs, we obtain an index from the summation of crosses of 22. The lower the index, the weaker the tuberculous process and the more massive the immunity.

Experiments

Passage and study of the proliferation of strain No 115 in guinea pigs. — In the course of four years, strain No 115 was passaged seven times in 43 guinea pigs. 15 animals were infected intravenously with 10 mg. Progress and details are presented in *Fig. 1*, which clearly shows that virulence was not increased either by passage from animal to animal or by infection with passage cultures. Tissular changes were minimal, in the spleen follicular hyperplasia, in the lungs and the liver scattered submiliary lymphocytic infiltrations, sometimes epithelioid cells were found, in one single case (guinea pig 349) was there an extensive focus. As to virulence, the culture grown from this animal (N 349) did not differ from the original strain.

All these changes were observed to persist until about the 136th day, subsequently they regressed. In guinea pig No 350 no characteristic histological changes were found 196 days following inoculation.

The dynamics of proliferation of the strain in guinea pigs is reflected by the curves in *Fig. 2*. Proliferation attained the peak 60 days after inoculation of 0.001 mg, but after 134 days large amounts were still present in the organs. 229 days following inoculation with 10 mg, strain No 115 could be grown (in guinea pig No 42), and was also obtained from guinea pig No 497, 274 days after passage of a culture from guinea pig No 448. These data reveal that strain No 115 grew actively in guinea pigs and remained alive for more than $7\frac{1}{2}$ months, which constitutes an important factor in immunization with living vaccine.

Immunogenic power of strain No 115. — The immunogenic power of strain No 115 was studied in guinea pigs in four experiments: in three experiments subcutaneously, in one by oral administration. The results are summarized in *Table I*. In all the animals immunized with strain No 115, less extensive tuberculosis was found to develop after infection with a virulent strain than

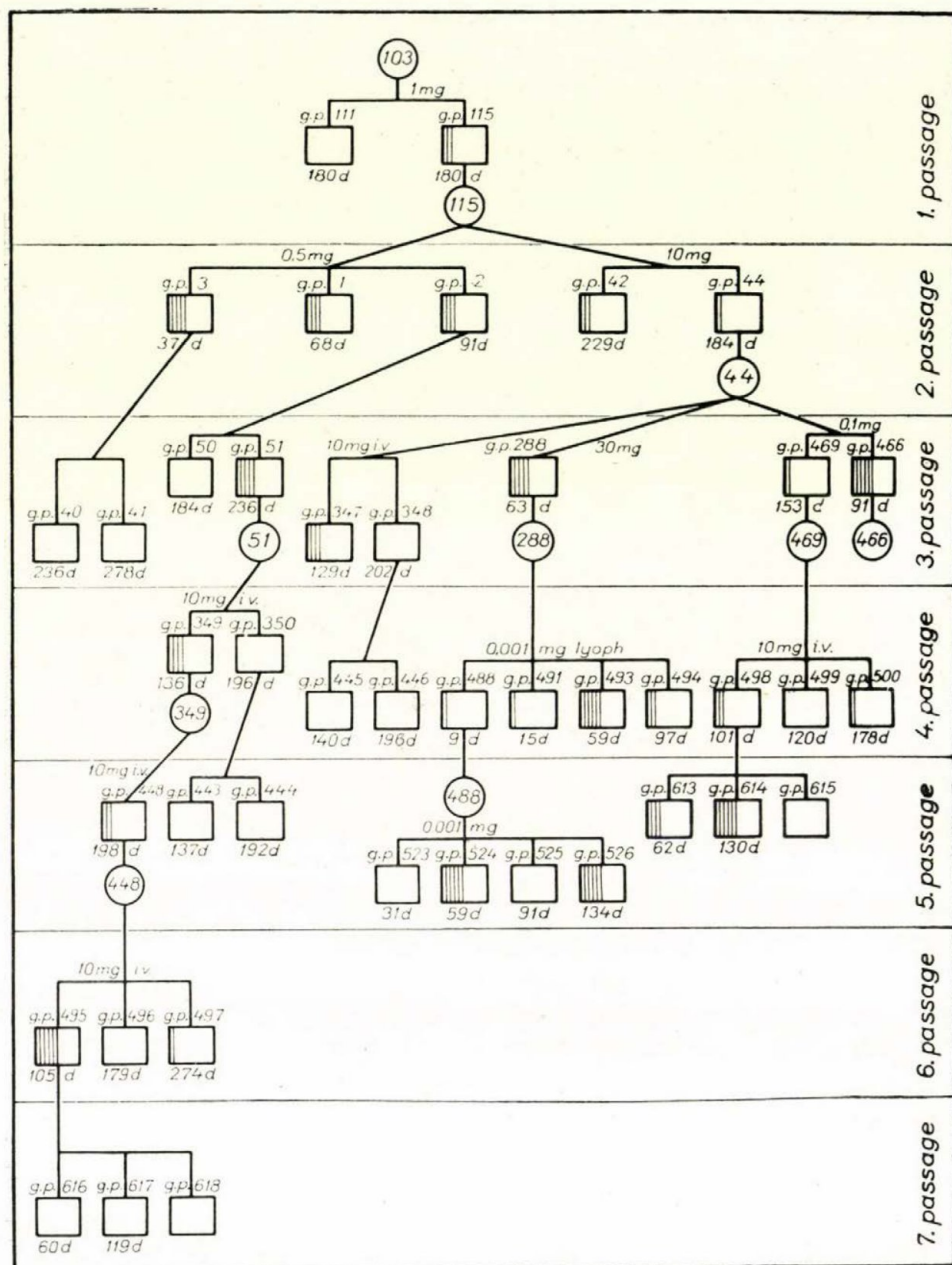


Fig. 1. Virulence of strain No 115 in guinea pigs in the course of passages

Signs: ○ = No. of strain
 □ = guinea pig
 g. p. = No. of guinea pig
 d = duration of passage, days
 ▨ = number of colonies grown from organs
 (1 line indicates 1 to 10 colonies;
 2, 11 to 20; 3, 21 to 50;
 4, 51 to 300; 5, more than 300 colonies)

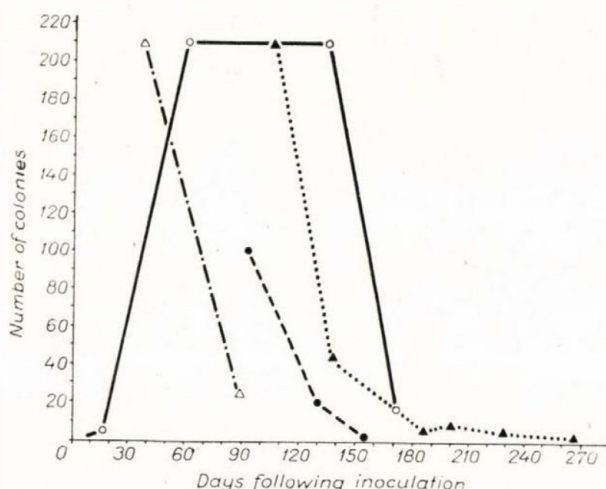


Fig. 2. Dynamics of proliferation of strain No 115.

Signs: Amount of inoculated bacteria

o—o = 0.001 mg

●—● = 0.1 mg

△-△ = 0.5 mg

▲.....▲ = 10 mg

in animals immunized with BCG. The indices demonstrating the results of immunization by the two strains do not show marked differences, but it must be taken into account that they regularly recurred in every experiment. Thus the immunity established by strain No 115 was of a higher order than that

Table I

Comparison of immunogenic properties of strain No 115 and BCG strain

Experiment	Strain	Route of inoculation	Number of guinea pigs	Tbc. index
I	115	subcutaneous	11	3.9
	BCG		12	4.9
	—		12	18.5
II	115	subcutaneous	12	7.4
	BCG		12	8.7
	—		12	17.3
III	115	subcutaneous	8	5.1
	BCG		9	7.6
	—		7	18.3
IV	115	oral	10	5.3
	BCG		10	6.8
	—		12	18.5

due to BCG. On this basis, and taking into account the data concerning the attenuated virulence, strain No 115 may be regarded as a vaccine strain.

Pathogenic properties of strain No 115 in mice, rabbits and monkeys. — The virulence of strain No 115 was investigated in white mice. Five animals were infected intravenously with 0.1 mg, and five with 1.0 mg. None of the animals died in consequence of inoculation. 32 to 133 days following inoculation the animals were sacrificed; pathologic changes of the organs were studied and the amount of living bacteria present in the organs was determined by cultivation. In the animals inoculated with 0.1 mg, focal pulmonary changes were observed after 67 days, and even 133 days following infection the great number of colonies growing on cultivation showed that the inoculated bacteria were in a state of active multiplication. In the animals inoculated with 1.0 mg, pulmonary changes were graver, but not lethal. Hypertrophy of the lymph nodes was also present. Cultivation yielded thousands of colonies. In relation to mice, the virulence of our strain was consistently higher than that of the BCG strain.

The pathogenicity of strain No 115 to rabbits was tested in three animals, injecting 20 mg of the culture intravenously. The animals weighed 1500 to 1850 g each. One rabbit died 23 days after infection. A great number of pulmonary foci were found. Instead of the normal 6 to 8 g, the lung weighed 58 g. The second rabbit was killed 35 days following infection. In this animal the pulmonary changes were more pronounced. On the lungs surface there were great numbers of foci, and the lungs weighed 70.2 g. Instead of the normal 1 g, the spleen weighed 10 g. The third rabbit was killed 56 days after infection. In this animal we observed partial healing of the pathological pulmonary change. The lung weighed 23 g, the spleen 6 g. Our findings show that strain No 115 possesses a certain virulence for rabbits, which is, however, lower than the virulence of BCG strains in relation to this species.

The pathogenic properties of strain No 115 were studied also in eleven monkeys (*Macacus cynomolgus*) whose weight varied between 1450 and 3800 g. 4 monkeys were inoculated with 20 mg of the culture, 3 with 10 mg, and 4 with 1 mg, either intravenously or subcutaneously. All animals were sacrificed 42 to 190 days following infection. Pathohistological investigation of their organs was performed by D. KARASSZON and G. GYENES. The gross changes are presented in *Table II*.

Our observations and the results of histological investigations may be summarized as follows:

(i) Two monkeys (Nos 54 and 55), each weighing 1500 g, were infected subcutaneously with 20 mg. One was killed 90 days, the other 134 days, following infection. 20 days after injection a subcutaneous abscess developed, which ulcerated. 60 days after infection, local changes regressed, leaving a scar. 90 days after infection autopsy revealed hyperplasia in the lymph nodes

Table II
Pathogenicity of strain No 115 in monkeys

Animal No.	Inoculation route, dose in mg	Experiment duration in days	Gross changes					
			site of inoculation	lymph nodes	spleen	liver	kidney	lungs
59	1 s. c.	92	+	—	—	+	—	—
61	1 s. c.	190	++	—	—	—	—	—
58	1 i. v.	92		—	++	—	+	+
60	1 i. v.	156		—	—	—	—	+
64	10 i. v.	42		—	—	—	—	+
62	10 i. v.	54		—	+	—	—	++
63	10 i. v.	121		—	—	—	—	+
55	20 s. c.	91	—	+	—	—	—	—
54	20 s. c.	134	—	—	—	—	—	—
56	20 i. v.	116		+	+	—	—	—
57	20 i. v.	134		+	—	—	—	++

Signs: — = no tubercle formation.

+

++ = 10 to 50 hardly discernible grey foci, abscess 3 by 2 cm at the site of inoculation.

and some lymphocytic foci in the liver and the lungs, but no caseation or calcification. 134 days following infection, a few small foci of epitheloid and lymphoid cells were detected in the spleen. The lungs showed diffuse histiocytic infiltration but no foci (*Fig. 3*).

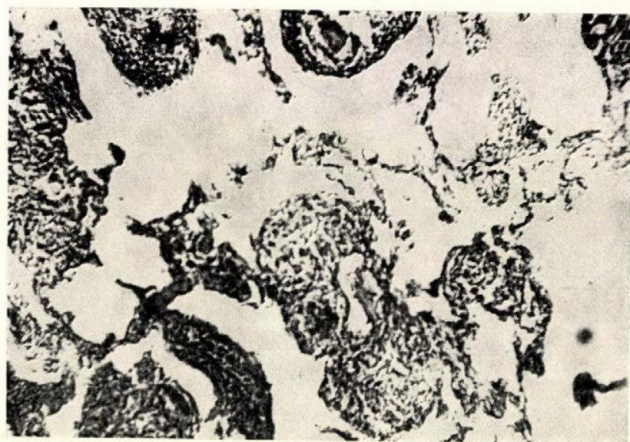


Fig. 3

(ii) Two monkeys (Nos. 56 and 57), weighing 2700 and 3000 g, respectively, were infected with 20 mg intravenously. This is the most serious route of infection, the whole organism being simultaneously invaded by a great amount of bacteria. One monkey was killed 116 days, the other 134 days, after the infection. In the first animal, the spleen, lung and lymph nodes displayed no specific change but a few lymphocytic foci in the liver. In the second monkey the spleen showed proliferation of lymphoid elements, in the lungs there were productive tuberculous infiltrations with lymphoid cells and histiocytes, without caseation (*Fig. 4*).

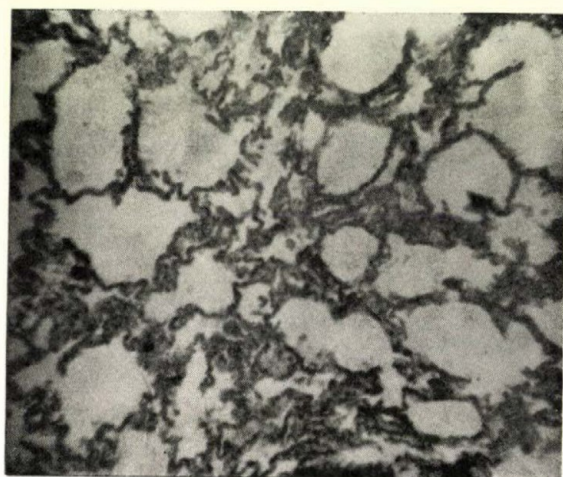
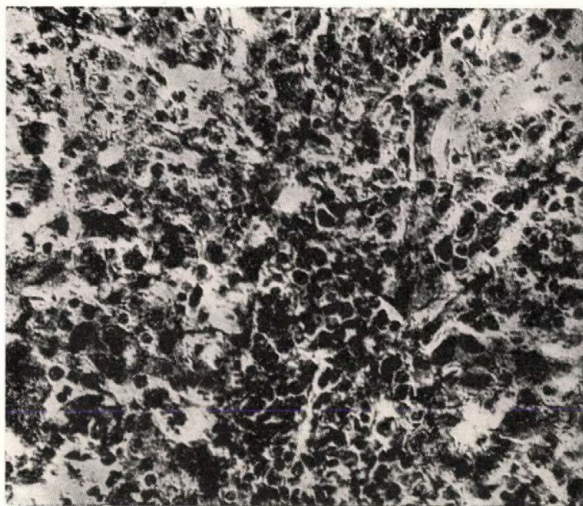


Fig. 4

In monkeys, where, according to our observations, 0.00001 mg of a virulent strain leads to the development of grave lethal tuberculosis in 90 to 120 days, 20 mg of our strain did not give rise to progressive changes after 134 days, only to certain non-specific tissue changes. After 134 days, the changes were somewhat graver than after 90 days, but still microscopic, and their further course would have doubtlessly ended in healing.

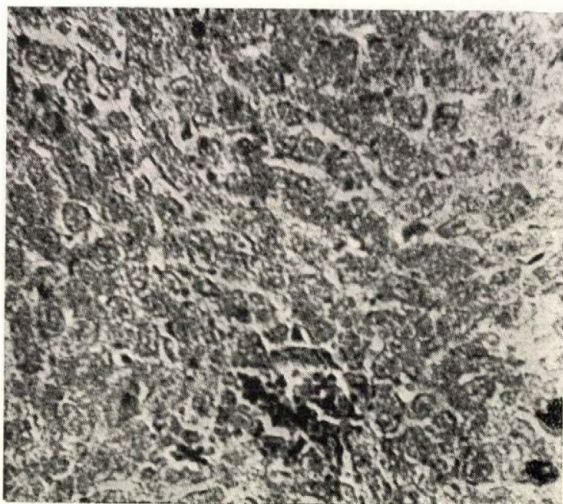
The animals infected with 1 mg doses yielded remarkable results. Two animals (Nos. 58 and 59) weighing 2100 and 2900 g, respectively, were sacrificed 92 days following infection, further two (Nos. 60 and 61) weighing 1470 g and 4250 g were sacrificed 156 resp. 190 days after infection.

In each of the subcutaneously infected animals an encapsulated abscess was found at the site of the infection. Ninety-two days after infection no histological changes were present in the lymph nodes or spleen. Scattered infiltration with histiocytes and monocytes was noted in the liver (*Fig. 5*).

*Fig. 5*

In the lungs, no focal infiltration was seen.

In the intravenously infected animal 92 days after infection only some slight diffuse epitheloid infiltrations were present in the lymph nodes, no changes in the spleen, diffuse lymphocytic infiltrations in the liver. In the pulmonary peribronchial and interalveolar tissue there was some proliferation of histiocytes and lymphoid elements. A few giant cells were also encountered (*Fig. 6*).

*Fig. 6*

One regular tubercle and some scattered proliferating foci of lymphoid cells were detected in the renal cortex.

Summing up our findings in the monkeys, in more than 6 months our strain even when given intravenously did not cause any progressive changes. The benign microscopic changes corresponded to the usual process elicited by vaccination and repeatedly encountered in guinea pigs under similar conditions. This means that the active multiplication of bacteria continues for 4 to 4½ months — as described before — and histological changes develop accordingly.

Acknowledgements: I wish to thank Dr. D. KARASSZON and Dr. G. GYENES for the performance of the histological examinations.

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Address of the authors: GYULA WEISSFEILER, VALENTINA KARASSOVA,
State Institute of Hygiene, Budapest IX, Gyáli út 2/6.

STUDIES ON THE INTERACTION BETWEEN COXSACKIE AND POLIOMYELITIS VIRUSES

IV. FURTHER EXPERIMENTS CONCERNING THE ANTAGONISM BETWEEN COXSACKIE B1 VIRUS AND THE LANSING STRAIN OF POLIOMYELITIS VIRUS IN YOUNG MICE

By

I. DÖMÖK

State Institute of Hygiene, Budapest

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Summary. Mice nine days of age were intracerebrally infected with 10^5 one-day-old-mouse-LD50 of C B1 virus and ten days later with 10 LD50 of type 2 poliovirus. In the subsequent 10-day period 3 or 4 mice were killed daily to titrate poliovirus in their CNS. No multiplication of poliovirus was detected.

C B1 virus could be re-isolated in the usual manner from the CNS of the double-infected mice, as well as from the mice infected with C B1 virus only, until the 18th day of C B1 infection. On the 43rd day after infection the C B1 virus was detected in a mixed pancreas-liver suspension of two mice which had seven days earlier been subjected to cortisone treatment and other procedures aiming at weakening resistance. It is suggested that the lasting interference with poliovirus demonstrated by us is due to the persistence of C B1 virus.

As described in previous papers [1, 2, 3], 9 day old mice when infected with Coxsackie (C) B1 virus develop lasting resistance against the Lansing strain of poliovirus. The degree of resistance was found to depend upon the time interval between the two inoculations. As measured by biometric-analytical methods based on the death rate due to poliovirus, and on the survival time, it was the highest between the 5th and 20th days following C infection, tended downwards thereafter, but remained significant for at least 60 days.

The present paper deals with the multiplication of poliovirus in C B1 virus-infected mice, and presents some data on the long persistence of C B1 virus in young mice despite the high titre of circulating antibody. The possible role of *occult* virus in maintaining resistance is discussed.

Materials and methods

Viruses. The C B1 strain and the mouse-adapted Lansing strain as well as the procedures employed in the preparation and storage of standard viral suspensions were those used by us previously [1].

Immune sera. The C B1 serum used in the experiments was prepared in mice weighing 10 to 12 g each. The schedule of immunization has been given in a previous paper [1]. The polio type 2 serum was kindly supplied by Professor H. PETTE (*Hamburg*).

Mice. Albino mice from the breeding stock of this Institute were used throughout. When C B1 virus was titrated, each dilution of virus was inoculated into 8, when re-isolation was attempted, each specimen was tested in 24 to 32 one-day-old mice. Each dilution of poliovirus was inoculated into 8 to 10 mice weighing 14 g each. For the interference experiments well-developed nine-day-old animals which had been observed from the day of delivery were used exclusively.

Preparation of the CNS and blood specimens of mice. The CNS specimens obtained from the groups signed I, II, and III in Table I were prepared as follows. The samples stored in

dry ice were melted by placing them into melting ice, ground with quartz sand, and diluted with saline to make 20 per cent suspensions. These were centrifuged with 4000 r. p. m. for 15 minutes, and the supernatants were used. In the experiments aiming at the re-isolation of C B1 virus polio type 2 serum was added to the supernatant and the mixture was inoculated into mice as soon as possible. While inoculation was being done, the material was kept at 0° C. The other specimens, after being mixed with serum, had been incubated at 37° C for one hour before inoculation.

Blood samples were allowed to clot. Serum was pipetted off, diluted 1 : 5 with saline, heated in sealed ampoules at 56° C for 30 minutes, and stored at +4° C until used.

Titres were computed according to REED and MUENCH's method [4].

Experimental

Nine-day-old mice were divided into three groups. As far as it was possible, litter-mates were equally distributed. Each animal in groups I and III received 10⁵ one-day-old-mouse-LD50 of C B1 virus in a volume of 0.02 ml, intracerebrally. Mice in group II were given a control suspension in the same manner. Ten days later the survivors in groups I and II were infected with 10 LD50 of poliovirus in 0.03 ml while those in group III received a normal brain suspension, intracerebrally. Six and 12 hours later, and every day in the following ten-day period, 3 or 4 symptomless mice, littermates if possible,

Table I

The schedule of titrations

Exper. No.	Group of mice	Materials tested	Test for	Incubation with equal volume of	Temperature	Time	Test mice
1 2	I	brain* cord*	Lansing virus	anti-C B1 serum 1 : 5 dil.	37° C	1 hr.	Adults weighing 14 g
3 4	II	brain* cord*					
5 6	I III	brain+cord* brain+cord*	C B1 virus	anti-Lansing serum 1 : 5 dil.	0° C	no incubation	One-day-old suckling
7 8 9 10	I III I III	brain + cord 20% susp. brain + cord 20% susp. serum 1 : 5 dil. serum 1 : 5 dil.	C B1 antibodies	C B1 standard viral susp.*	37° C	1 hr	One-day-old suckling
11	normal mice	brain + cord 20% susp.	Virus control	C B1 standard viral susp.*	37° C	1 hr	One-day-old suckling

* Tenfold dilution series from 20 per cent suspension.

were killed by bleeding under ether narcosis. The brains as well as the spinal cords of the simultaneously killed mice were collected by group and stored in sealed ampoules until used. Blood was taken from the mice in groups I and III on the 10th and 20th days of the experiment.

The schedule of examination of the samples collected is presented in Table I. Each mouse received 0.02 ml, subcutaneously when C B1 virus, intracerebrally when poliovirus, was attempted to be isolated. The animals given C B1 virus were observed for 15 days, those infected with poliovirus for 30 days.

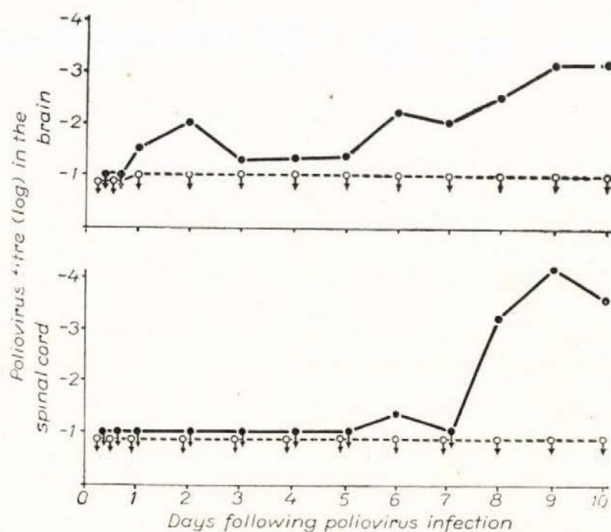


Fig. 1. Multiplication of poliovirus in the CNS of the mice in groups I and II.

- — — — ○ Titre in the mice previously infected with C B1 virus (Group I)
- — — — ● Titre in the control animals (Groups II)

Multiplication of poliovirus in the CNS of mice inoculated ten days earlier with C B1 virus. The dynamics of virus multiplication in the brains and cords in group II was as expected (Fig. 1). Virus multiplication was demonstrated in both the brain and the cord; first in the brain, but the peak of titre in the cord was higher ($10^{-4.2}$) than in the brain ($10^{-3.1}$). Unlike this, poliovirus could be demonstrated only on certain days in the brains and cords obtained from group I (mice pretreated with C B1 virus). One or two mice were found to be paralysed in each of the groups inoculated with one of the 10 per cent brain suspensions prepared from the mice which had been killed 12 hours, 1, 4, 5, 8 and 10 days, respectively, after infection with poliovirus. Among the 10 per cent spinal cord suspensions virus was demonstrated in those obtained in the 6th hour (3 of the 8 mice showed paralysis) and in the 6th and 9th days (one of the 8 mice showed paralysis in each case).

Thus poliovirus showed no multiplication in the CNS of mice 10 days earlier inoculated with C B1 virus, *i. e.* the latter virus had induced a complete interference.

The C B1 virus and specific antibody content of the CNS of mice inoculated at 9 days of age with C B1 virus. Table II shows the titres of C B1 virus and those of the antibody titres in the serum samples, taken from groups I and III during the period when interference was established. Virus was demonstrated despite the high titres of antibody in the CNS suspensions from group I and group III. The 10 per cent CNS suspensions obtained from group I yielded virus every day from the 10th to the 16th and on the 18th day following C B1 infection. In group III virus was demonstrated in the CNS samples on the 10th, 11th, 13th and 15th days. Attempts to infect mice with diluted suspensions generally failed. Although there was some difference between groups I and III in yielding virus, this was not significant in view of the sources of error due to the presence of antibody.

Table II

C B1 virus and antibody in the CNS of mice from the 10th to 20th day following infection

Days after C B1 infection	Virus		Antibody		Serum antibody	
	in the 10% susp. of CNS					
	Group I	Group III	Group I	Group III	Group I	Group III
10	7/51*	13/64*	2.5**	2.0**	4.5**	4.1**
11	4/30	4/32	1.8	1.9		
12	1/32	0/32	2.0	2.5		
13	1/24	4/30	1.7	1.9		
14	4/32	0/24	2.7	2.5		
15	4/24	1/24	1.9	2.3		
16	4/26	0/24	1.8	2.0		
17	0/24	0/32	1.8	1.8		
18	2/32	0/32	2.2	1.9		
19	0/30	0/26	2.2	1.7		
20	0/32	0/32	1.8	2.3	4.4	4.4

Mice in group I were superinfected with poliovirus on the 10th day.

* = Numerator, mice showing symptoms of C B1 infection.
Denominator, mice inoculated.

** = Neutralization index, log.

To present evidence that the C B1 virus was recovered from group I on the 18th day, and that from group III on the 15th day, the brains of the mice which had been infected with the corresponding materials and had shown symptoms of meningoencephalitis were suspended in saline, and an aliquot

of each suspension was mixed with anti-C B1 serum; the mixtures were incubated at 37° C for one hour and inoculated into suckling mice. By this experiment both agents proved to be C B1 virus. Thus, the virus was present in the CNS of the mice in spite of the high level of specific antibody on the 18th and 15th days following infection while multiplication of the challenge poliovirus, as demonstrated previously is suppressed by the interference phenomenon.

Attempts to demonstrate C B1 virus in a later period of infection. We have attempted to re-isolate the C B1 virus from the CNS of mice inoculated at 9 days of age 30 and 36 days after the mice had been infected. These attempts, though they involved great number of mice for re-isolation, failed when conducted in the usual manner. Nevertheless, we succeeded in demonstrating the presence of the virus in this period by the following experiment.

Thirty-six days after inoculation with C B1 virus ten mice were submitted to the following treatments. 0.25 ml blood was withdrawn from each animal by heart puncture, and this was followed by subcutaneous injection of 10 mg cortisone divided into four equal doses in a volume of 0.1 ml each, administered at 1, 5, 25 and 29 hours following heart puncture. In the 25th hour in addition to the cortisone treatment each mouse was given 0.02 ml saline intracerebrally. Eight of the mice had succumbed within 7 days and, in spite of each CNS sample having been tested in 16 one-day-old mice, virus could not be recovered. The survivors, hypokinetic and with ruffled fur on the 7th day, were bled out under ether narcosis, and after attempting to remove the residual antibody by infusing saline through their hearts, the CNS, liver and pancreas were tested for C B1 virus. In the CNS we failed to demonstrate virus even by two blind passages in suckling mice. Of the 16 one-day-old sucklings, however, which had been inoculated with a mixed suspension of liver and pancreas five developed symptoms of encephalitis after an incubation period of 8 to 10 days. The virus recovered from the five sucklings was neutralized by anti-C B1 serum.

Discussion

The study of interaction between the members of the Enterovirus family has been particularly promoted by the introduction of large-scale vaccinations with attenuated poliovirus strains. It has been demonstrated that the occurrence of certain types of C and ECHO viruses may interfere in the intestinal tract with the multiplication of the attenuated strain, thus, with the immunizing effect of the vaccine [10]. Other strains seem to stimulate the mild effects of the attenuated strain in monkey experiments [5].

The interference by the polioviruses with the multiplication of C B viruses has been studied by LYCKE [6] in tissue cultures. In experi-

ments carried out in mice the inhibition of the symptoms due to poliovirus served as the indicator of interference between CB and polioviruses [7-9]. In the previous studies of this series we calculated the degree of interference from the lower mortality and the prolonged survival times [1, 2, 3]. On these grounds the potency of poliovirus in mice inoculated with C B1 virus 10 days before was found 0.4 to 0.8 per cent of that established in the control animals. Based on the present experimental results it seems reasonable to ascribe this resistance to the inhibition of the multiplication of poliovirus by the interfering virus.

We have demonstrated that the increased resistance which follows C B1 virus infection is of long duration. It was pointed out that analogous phenomena had been observed in the case of other pairs of viruses. More recently, when studying interference between the viruses C B1 and C A14, WEIGAND has obtained results essentially identical with ours [11]. Our present results seem to be satisfactory to suggest that the interference described in our previous papers is caused by the long persistence of the interfering virus. Until the 18th day following infection the virus could be detected by the usual infectivity test whereas re-isolation of the virus in a later period needed some special procedures including administration of excessive doses of cortisone to, and removal of antibody from the host. We have no satisfactory information on the location of the persistence, and on the circumstances making the virus capable of surviving despite the high level of circulating antibodies. As demonstrated in our experiments on the inhibition of the multiplication of poliovirus, the interference phenomenon under discussion resides in the CNS. Thus, the CNS must be at least one of the organs where the virus can persist. In spite of this, on the 43rd day of infection we succeeded in demonstrating virus in a mixed liver-pancreas suspension, but not in the CNS. BORING *et al.* [12] have demonstrated that in cortisone-treated adult mice C B1 virus reaches the highest viral titres in the liver and in the pancreas. In the experiments of these authors the maximum titre of virus reached in the brain was 3 and 2 log units lower than in the pancreas and in the liver, respectively. These findings have lead us to conclude that in our animals the excessive doses of cortisone had made the liver and the pancreas capable of supporting the multiplication of virus to a demonstrable concentration.

ACKERMANN has demonstrated that the action of antibody upon an infected tissue culture is not to eliminate the homologous virus but rather to contribute to its survival [13]. Such a host-virus equilibrium might have developed in our nine-day-old mice.

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Address of the author: ISTVÁN DÖMÖK,
State Institute of Hygiene, Budapest IX. Gyáli út 2/6.

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STUDIES ON BENZOATE-RESISTANT YEASTS*

By

M. INGRAM

Low Temperature Research Station, Cambridge

(Received September 23, 1959)

Summary. Several strains of yeasts isolated from benzoate-preserved citrus beverages were found to resemble *Saccharomyces acidifaciens*. They were demonstrated to resist 500 mg of benzoic acid at pH's below 3. Assimilation and respiration experiments showed that they possess a limited ability to break down benzoic acid. A pathway via *p*-hydroxybenzoic acid, protocatechuic acid, catechol and β_4 -ketoadipic acid was indicated.

During the hot summer of 1955, there were serious outbreaks of fermentation in commercial fruit squashes prepared from citrus fruits. These squashes contained about 500 mg/l of benzoic acid — the statutory limit in Britain is 600 p. p. m. — and some of them had a pH near 2.5, yet they nevertheless fermented. The significance of this low pH lay not so much in the value itself, which is not sufficient to prevent yeast growth, but rather in the fact that at such a pH benzoic acid is almost wholly undissociated in solution.

Hence it must be supposed that the fermenting organism resisted 500 p. p. m. of undissociated benzoic acid, in contrast with levels of 10–50 p. p. m. for normal yeasts [c. f. 5]. This extraordinary resistance to benzoic acid, at least 10 times the usual, suggested that the yeasts might be able to break down benzoic acid. The following investigation was undertaken to test the above suppositions.

Materials and methods

Three isolates from fermenting squash were obtained: called LTSI 239, 241 and 242. For comparative purposes a type culture of *Saccharomyces acidifaciens* which had not known previous contact with benzoic acid was selected: this was no. 685 from C. B. S. Delft.

Cells were grown and aerated by shaking in T-tubes, using the T-tube method of BARNETT and INGRAM [2]. Cell densities were measured on an EEL nephelometer previously calibrated in terms of dry weight/ml.

The basal medium, A₂, the synthetic mineral salts medium described by BARNETT and INGRAM [2], contained trace elements and vitamins. To the trace element + salts was added the C source and/or benzoate to the required concentrations, the pH was adjusted and the solution autoclaved at 15 lbs. for 15 mins. Vitamins were added aseptically to the cooled solution. In the case where sugars were suspected of significant decomposition by autoclaving (as at 30% concentration), the whole medium, salts/TE vitamins, C-source and benzoate were Seitz-filtered. Sodium benzoate was prepared by neutralizing "AnalaR" benzoic acid

* Read at the 2nd Congress of the Hungarian Association of Microbiologists, September 23, 1959, Budapest.

with „AnalaR” NaOH collecting the product after evaporation and recrystallizing. All other materials and reagents were „AnalaR” grade.

The Rothera test was performed by both the old and new methods [3]. Oxygen uptakes in the Warburg manometer were measured by the accepted techniques [10]. Where necessary, cell suspensions were analysed for intermediates after the removal of cells by passing the suspension through a No. 4 sintered glass filter.

Benzoic acid was estimated spectrophotometrically at 225 m μ where it gave a broad but pronounced absorption peak in Medium A₂ (the peak is normally at 227 m μ).

Results

The identity of the isolates. The causative yeasts were isolated separately by the firms concerned. Nevertheless, in morphology and principal biochemical properties they correspond fairly well with a type strain of *Zygos. mandshuricus* which was placed by LODDER and VAN RIJ [7] in *Sacch. acidifaciens* (Table I): further as will be seen, they are acidophilic, and share other properties.

Table I
Standard fermentation (F) and C-assimilation (A-auxanogram, T-tube) reactions

	CBS 685			239			241			242		
	F	A	T	F	A	T	F	A	T	F	A	T
Glucose	(+)	(+)	+	+	+	+	+	+	+	+	+	+
Galactose	(-)	(±)	.	-	-	+	-	-	.	-	-	.
Sucrose	(-)	(-)	-	-	-	+	-	-	-	-	-	-
Lactose	(-)	(-)	-	-	-	-	-	-	-	-	-	-
Maltose	(-)	(-)	-	-	-	-	-	-	-	-	-	-
Melibiose.....	.	.	-	-	.	-	-	.	-	-	.	-
Raffinose	-	.	.	.	.	-	.	.	+	.	.	.
Arbutin		(-)			-			-			-	

() data from LODDER & VAN RIJ [7], confirmed
 . test not completed

The strains are probably not absolutely identical, however, even discounting odd results with sucrose and raffinose; for extensive assimilation tests reveal minor differences (Table II) in utilization of erythritol, sorbose and trehalose. A common source thus seems unlikely.

The resistance of the yeasts to benzoate. It was essential to confirm that the isolates had the indicated resistance to benzoate. The first experiments (Table III) were made at pH 5, near the normal pH optimum for yeasts. It is clear that in the presence of 10 g/l sucrose the isolates grew with 1000 mg/l benzoic acid, i. e. 560 p. p. m. undissociated. With no sugar, growth of the isolates was

Table II
C-assimilation reactions (T-tubes)

	CBS				CBS		
	685	239	241		685	239	241
arabinose	—	—	—	glycerol	.	+	+
rhamnose	—	—	—	erythritol	—	+	—
ribose	—	—	—	adonitol	+	+	+
xylose	—	—	—	mannitol	+	+	+
				sorbitol	+	+	+
sorbose	—	+	—				
				lactic ac.	—	—	—
cellobiose	—	—	—	fumaric	—	—	—
trehalose	+	—	—	malic	—	—	—
Me glucoside	—	—	—	succinic	—	—	—
				citric	—	—	—
melezitose	—	—	—				
				glutamic	—	—	—
gluconolactone	+	+	+	proline.....	—	—	—

doubtful; but type strain 685 was plainly inhibited, though with sugar it tolerated 100 and maybe 500 mg/l.

Table III

Growth (as nephelometer reading) on synthetic medium with C-source benzoic ac. (100, 500, 1000 mg/l) or with the same plus 1% sucrose (autoclaved) pH 5.0 (benzoic 56% undissoc. ac.)

Days	CBS 685			239			241			242		
	100	500	1000	100	500	1000	100	500	1000	100	500	1000
Benzoate alone												
0	6	5	6	6	6	6	6	5	5	6	5	6
2	6	5	5	11	8	7	10	6	4	11	7	6
3	no change			13	9	6	15	8	5	19	7	7
5	”	”		15	10	6	17	15	5	38	19	7
7	”	”		15	12	7	17	14	6	.	.	12
Benzoate + sucrose												
0	6	6	5	6	6	6	5	6	5	6	6	5
2	6	5	6	31	18	14	26	20	10	32	12	7
3	6	5	7	36	19	17	25	23	17	37	18	13
5	20	15	12	40	29	17	31	26	20	41	23	16
7	25	.	11	52	37	28	27	39	28	43	32	14

It seemed desirable to extend the results to cover more acid conditions and higher sucrose concentrations, to imitate conditions in fruit squash (*Table IV*). Growth proved to be generally better at pH 2.5 than 3.5 which

Table IV

The influence of pH and sucrose concentration and benzoic acid (500 mg/l) on growth (nephelometer readings) in a synthetic basal medium

Days	CBS 685					239					241				
	0	1	30	10	300	0	1	30	10	300	0	1	30	10	300
<i>pH 2.5</i>															
0						6	7	5	5						
2						6	7	12	74						
4						9	9	49	∞						
8						12	9	60							
<i>pH 3.5</i>															
0	5	4	4	5	5	4	4	4	5	5	5	4	5	5	5
2	5	4	10	36	71	5	7	22	37	∞	5	4	26	52	∞
4	5	4	43	50	∞	5	28	51	53	.	5	14	73	76	.
7	5	25	51	60	.	5	31	53	82	.	5	29	72	93	.
<i>pH 4.5</i>															
0	4	4	5	5	4	4	4	4	10	4	4	5	4	5	5
2	4	7	9	14	57	4	8	28	29	99	4	6	26	18	99
4	4	8	32	20	∞	4	11	43	35	∞	4	9	41	31	∞
7	4	8	33	23	.	7	11	51	35	.	5	9	49	31	.
. No observation made. 0=500 mg/l benzoic ac. 1=do + 10 g/l sucrose 30=do + 300 g/l sucrose															
∞ offscale (>120) 10=10 g/l sucrose 300=300 g/l sucrose															

was better than 4.5; and 30% sucrose gave much better growth than 1%. The stimulatory effect of sugar on resistance to benzoate at 500 mg/l was confirmed: virtually no growth took place with no sugar; there was sometimes a little with 1%; all strains grew with 30% even CBS 685 at pH 3.5. This confirms that all these yeasts, including the type strain, can tolerate 500 p. p. m. undissociated benzoic acid.

Disappearance of benzoate. The first tests were simply made manometrically, to see whether the yeasts were capable of oxidizing benzoate. Because it seemed likely that adaptation might be involved, comparative experiments were made with cells grown in the synthetic medium + glucose with or without 500 mg/l benzoic acid. Results are in *Table V*. The glucose respiration of

the cells not exposed to benzoate was largely suppressed in the exposed cells; but the latter were evidently able to oxidize benzoate in the presence of glucose, though not in its absence (cf. growth experiments *Table I*). There was clearly an interrelation of substrate concentrations (and it may be added of pH), which await exploration. Unfortunately, because there was an extra O_2 uptake with benzoate only in presence of sugar, it was not sure whether the benzoate was merely stimulating glucose oxidation, though this seemed unlikely; and hence no deductions about the possible quantity of benzoate destroyed were possible. A direct attempt to measure that quantity had therefore to be made.

Table V

The ability to respire benzoate, in the presence and absence of glucose

(Cells of 239 grown on A2G + or - 500 mg/l. benzoate.
Washed and suspended in 0.1 M KH_2PO_4 at 25° C)

Substrate (M)		QO ₂ of cells grown	
		without benzoate	with benzoate
0 (endog.)		5	7
benzoate	10 ⁻³	4	9
	10 ⁻²	3	6
glucose	10 ⁻³	23	10
	10 ⁻²	74	17
glucose + benzoate			
10 ⁻³	10 ⁻³	23	13
10 ⁻²	10 ⁻³	90	25
10 ⁻²	10 ⁻²	74	38

Information from manufacturers suggested that fermented squashed contain somewhat less than the expected benzoate concentration. This might be (i) because a low benzoate concentration, caused by inadequate mixing, encouraged the fermentation; or (ii) because the yeasts destroy the benzoic acid. The strict control methods employed by the squash manufacturer, for legal reasons, encourage the second rather than the first suggestion. The postulated ability of the yeasts to destroy benzoic acid might obviously be related to their resistance to that compound.

In investigating this subject, there is a major difficulty with methods of estimating benzoic acid, which are laborious and not sufficiently specific. The medium used seemed likely to contain substances able to interfere. The colorimetric method recommended by SNELL proved laborious, insensitive and inaccurate. Hence U. V. absorption at 225 m μ was tried.

An experiment at pH 2.5 revealed a fall in optical density (O. D.) for 1—2 days, equivalent to a fall in the concentration of benzoic acid in the medium from 500 to 400—350 mg/l, corresponding roughly with commercial experience. But this fall in O. D. was followed by a rise (*Fig. 1*). On the basis of work by SISTROM and STANIER [9], R. B. CAIN suggested that this latter absorption might be due to the lactone of *cis-cis* muconic acid. Examination of the whole absorption spectrum revealed, in fact, development of a peak at

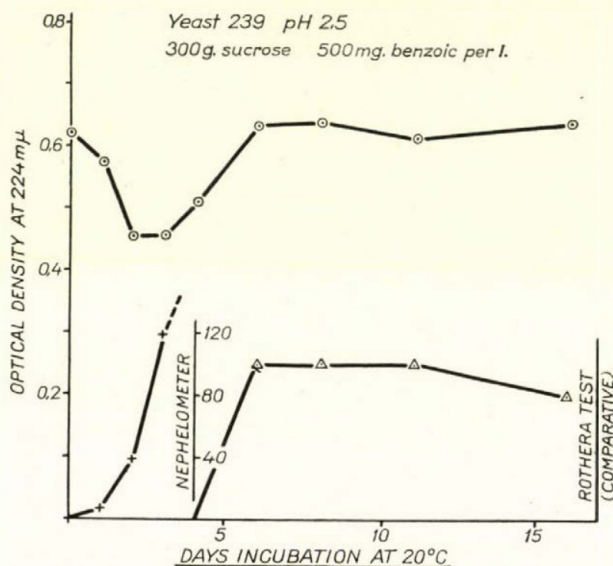


Fig. 1. Changes in optical density of the (cell-free) medium (A2) during growth of a benzoate-resistant yeast, in relation to growth of yeast and development of positive Rothera reaction.

230 $m\mu$, corresponding to that of the lactone (*Fig. 2*). Hence attempts were made to separate the components by extraction with petrol ether: diethyl ether, in which benzoic acid is soluble but the lactone probably is not [cf. 9]. This gave (*Fig. 3*) a diminishing O. D. in the extract (*i. e.* benzoic acid) with rising O. D. in the residue (presumed lactone), suggesting conversion of benzoic acid to the lactone. Knowing the two specific extinctions (that of the lactone is comparatively high) one can calculate the extent of the presumed conversion, which comes out at a reasonable value near 10%.

The route of breakdown of benzoic acid. The above-mentioned rise in O. D. at 230 $m\mu$ was followed 1—2 days later by the appearance of a positive reaction in the Rothera test (cf. *Fig. 1*). This was presumed to be due to β -ketoadipic acid [4], though the reaction is also given by other β -ketoacids notably acetoacetic and by acetone.

Ether extracts taken up in water gave a very weak reaction, eliminating any likelihood that the substance responsible for the reaction was acetoacetic

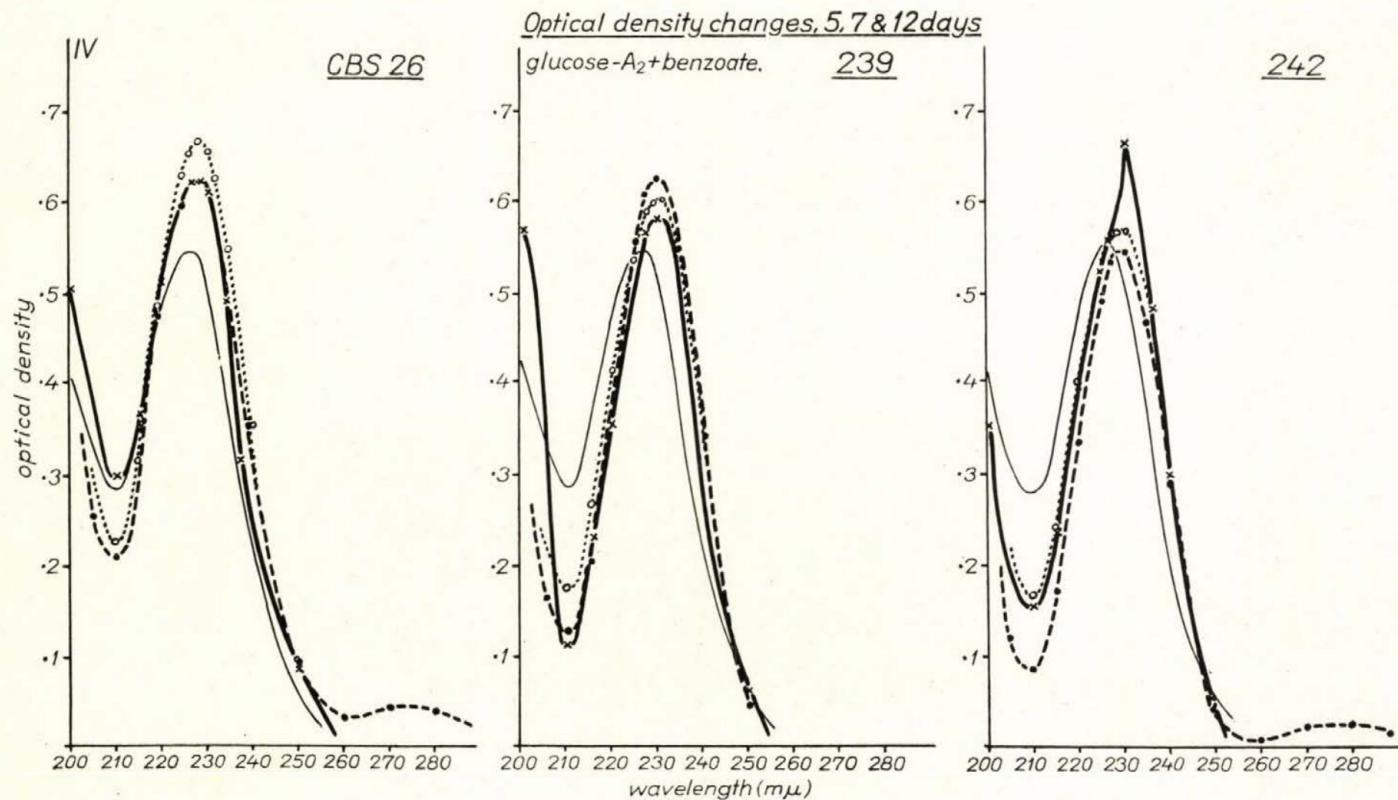


Fig. 2. The ultra-violet absorption spectra of the (cell-free) medium (A₂) growth of benzoate-resistant yeasts
— original medium, x—x 5 days, ·—·—· 7 days, o· · · o 12 days

acid or acetone. To confirm its identity, we took an 8 day culture giving by then a strong Rothera reaction, converted the acids to the 2,4-dinitrophenylhydrazones and extracted with ether/chloroform (after ISHERWOOD and CRUCKSHANK [6]). The extracted hydrazones were chromatographed on paper in tertiary amyl alcohol/propyl alcohol/ NH_3 65/5/30 and spots detected under U. V. light. The extract was compared with β -keto adipic acid synthesi-

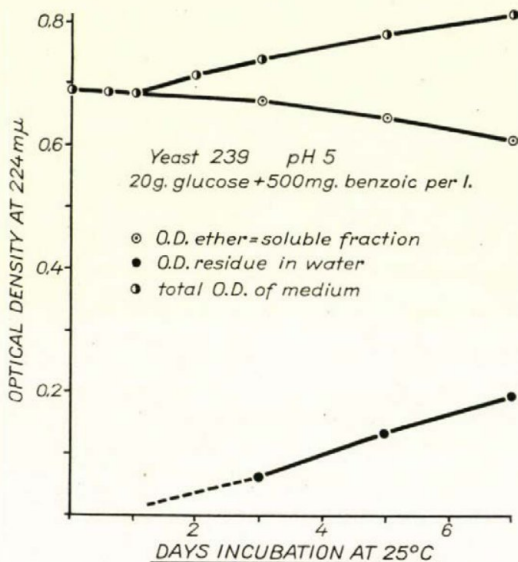


Fig. 3. Changes in the ultra-violet absorption by petrol ether/ether extracts (benzoic acid?) and by the residue (*cis-cis* muconic acid lactone?) of the (cell-free) medium (A2) during growth of a benzoate-resistant yeast

zed by the method of BARDHAN [1] and treated similarly. Two spots appeared, with the same R_F values for the extract and the reference spot (Table VI); so there is a strong indication that β -keto adipic acid was produced *via* the lactone from benzoic acid.

Table VI

Identity of Rothera-positive substance
RF value of 2,4-dinitrophenylhydrazones

	β -keto adipic acid	Culture extract
Spot I	0.868	0.870
II	0.526	0.521

Second spot possibly due to laevulinic acid produced during preparation of phenylhydrazones.

In an endeavour to identify other intermediates in the system, suggested by the work of SHEPHERD and VILLANUEVA [8] on *Aspergillus*, yeast 239 was grown on synthetic media containing catechol, *p*-hydroxybenzoic acid or protocatechuic acid, also a parallel test was made with benzoic acid. For comparison, a glucose control (no aromatics) and an uninoculated benzoic acid control were set up. The concentration of each of the above aromatics was 500 mg/l.

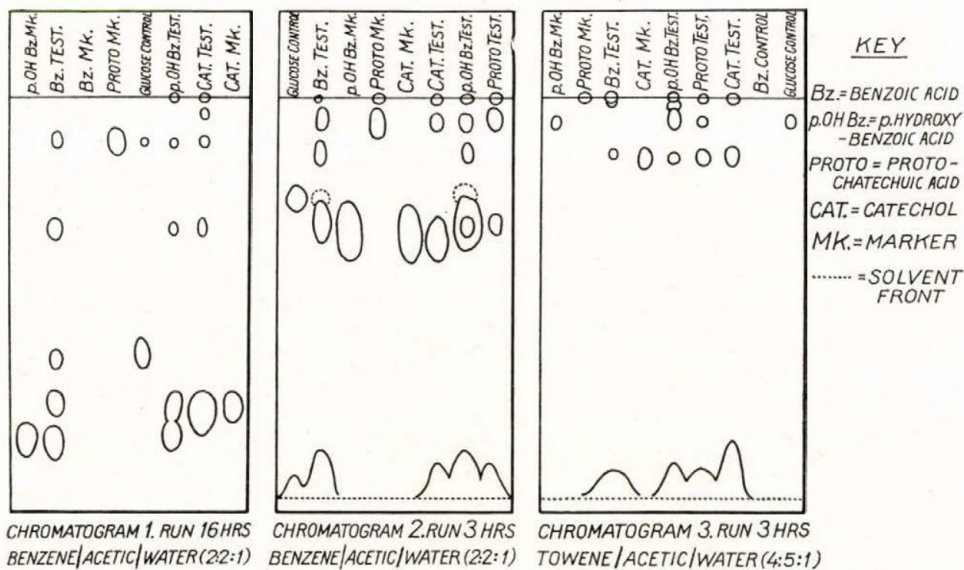


Fig. 4. Summary of chromatograms obtained from extracts of synthetic medium (A2) in which a benzoate-resistant yeast had been grown in presence of derivatives of benzoic acid

The medium after incubation was centrifuged to remove the cells, acidified to pH and extracted with $10 \times \frac{1}{4}$ volumes of diethyl ether. The ether extracts were combined and evaporated to dryness under reduced pressure, oxygen being displaced with nitrogen (to prevent oxidation of the aromatic). The residue was dissolved in 0.5 ml of alcohol and spotted on chromatograms; which were developed with one of two solvent systems — benzene/acetic/water or toluene/acetic/water — which have different relative R_F values for the substances under investigation. The chromatograms were sprayed with a diazotising reagent (*p*-nitraniline, hydrochloric acid, sodium nitrite).

On these chromatograms (summarized in Fig. 4) the extract from the test with benzoic acid showed spots corresponding to protocatechuic acid, *p*-hydroxy-benzoic and catechol, not seen in the benzoic acid control. (The glucose control showed a faint spot corresponding to protocatechuic acid, probably due to a small amount carried over on inoculating and concentrated in the extraction procedure; a second spot on the glucose control was proba-

bly due to a vitamin *p*-aminobenzoic acid added to the medium.) All three of the suspected intermediates also generated additional compounds: the extract from the medium containing *p*-hydroxybenzoic acid showed spots corresponding to protocatechuic acid and catechol; the medium initially containing only protocatechuic acid contained catechol and *p*-hydroxybenzoic acid; further, the catechol medium showed protocatechuic acid and a trace of *p*-hydroxy-

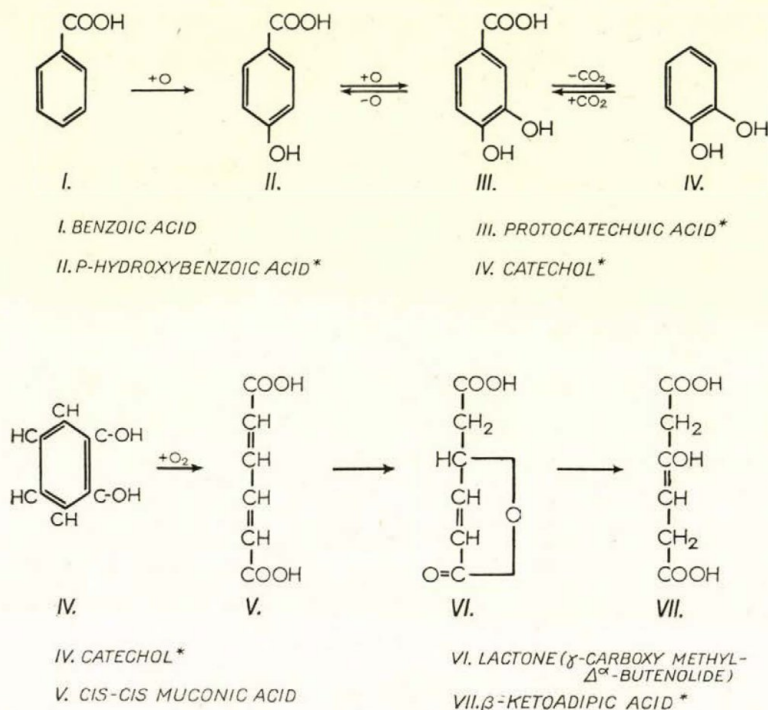


Fig. 5. Indicated reaction sequence for the breakdown of benzoic acid by a benzoate-resistant yeast

benzoic acid. Finally, running with the front in extracts from medium containing any one of the aromatics, was a brown substance not seen in either the glucose or benzoate control. It gave an intense blue colour with the diazo reagent. From its solubility and colour, it is suspected to be *o*-quinone: however, it is possible that the compound was produced by oxidation of catechol by the air.

At least, the yeast produces from benzoic acid substances that are chromatographically similar, in two different solvents, to *p*-hydroxybenzoic acid, protocatechuic acid and catechol; both oxidative and reductive steps are here

* Intermediates presumptively demonstrated

indicated, also carboxylation and decarboxylation. Hence it appears that the pathway for the breakdown of benzoic acid by the yeast may be as in *Fig. 5*. To the best of our knowledge, this sequence has not hitherto been suggested for yeasts.

Discussion

The investigation is far from satisfactorily completed. We have only the presumptive chromatographic evidence of the identity of our suggested intermediates; and none of the inferred enzyme systems has been demonstrated to be present in the yeasts. Most of the work has been done with only one of the strains isolated; whereas, besides the other isolates, it would be interesting to examine the type strain. We do not know why only a fraction of the total quantity of benzoate disappears, nor why sugar must apparently be present. Until these matters have been more fully explored, there is little reason to suppose that the limited ability of these yeasts to break down benzoate is related to their ability to resist much greater concentrations of it. If that were the case, we might expect them to be unusually resistant likewise to the postulated intermediates, such as *p*-hydroxybenzoate and catechol, which also remains to be tested.

Acknowledgements. Most of the work here described was carried out by Mr. R. B. CAIN or Mr. P. J. WILLIAMS, during their tenure of Vacation Studentships at the Low Temperature Research Station.

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Address of the author:

M. INGRAM

Low Temperature Research Station, Downing Street, CAMBRIDGE, England

HISTOPATHOLOGICAL FINDINGS IN CATS INFECTED WITH *ENTAMOEBIA HISTOLYTICA**

(A PRELIMINARY REPORT)

By

T. BAKÁCS and MARIA JANKÓ

State Institute of Hygiene, Budapest

(Received October 21, 1959)

Summary. Kittens were infected intracoeally with *E. histolytica* isolated from the faeces of children showing clinical symptoms and laboratory evidence of amoebiasis. The infected animals were kept under clinical and laboratory observation for half a year. Histological examination revealed characteristic changes especially in the colon, in the form necrotic areas in the mucosa, oedematous swelling and round cell infiltration in the submucosa. There were numerous *E. histolytica* in the intestinal glands, in some cases also in the spleen.

Ever since WALKER and SELLARDS' experiments [1] the significance of *E. histolytica* and especially its pathogenicity under the temperate zone has been studied by numerous authors of whom let us mention BRUMPT [2], CRAIG and FAUST [3] KUENEN and SCHWELLENGREBEL [4], HOARE [5], MATEVOSSIAN, SARKISSIAN and MARKARYAN [6]; and the Hungarian authors LŐRINCZ [7], MAKARA and ALMÁSY [8], MAKARA, VARGA and ZIEGLER [9], and VÉGHÉLYI [10]. The present series of experiments has been started by ZOLTAI and JANKÓ.

In 1953 ZOLTAI and JANKÓ found that in Budapest 1–1.5 per cent, in country towns and villages 10–12 per cent of the healthy children were excreting *E. histolytica*. The corresponding figures for healthy adults were 2.5 resp. 10.8. Between 1953 and 1955, examinations carried out at *László Hospital Budapest*, revealed that 20 per cent of the patients admitted with dysenteric symptoms were positive for *E. histolytica*. Their clinical observations showed that simultaneous infection with *E. histolytica* and *Shigella* caused more severe disease than *Shigella* alone. 1955 rectoromanoscopic examination revealed a 33 per cent incidence of *E. histolytica* in a selected group of outpatients. The clinical observations and animal experiments performed by ZOLTAI, JANKÓ, BALLÓ and LÓRÁNT between 1957 and 1959 clearly demonstrated the important role of *E. histolytica* in colitis. In these experiments cysts isolated from 163 cases were measured and data regarding the size of cysts and severity of the clinical picture were compared. No definite correlation was found, as symptomless carriers were observed among persons excreting large cysts and, alternatively, small cysts were often found in cases with severe colitis.

* Read at the 2nd Congress of the Hungarian Association of Microbiologists, September 23, 1959, Budapest.

These results were in agreement with the opinion of WENYON [11] and MELENEY and FRYE [12] and others.

Considering these results, it seemed interesting to study the pathogenicity for animals of the *E. histolytica* strains occurring in Hungary.

Materials and methods

The cultures to be inoculated were obtained from the faeces of children showing clinical symptoms and laboratory evidence of *E. histolytica* infection. On the basis of their clinical symptoms the patients were divided into 4 groups according to CRAIG's well-known classification, viz. (i) Symptomless "carrier"; (ii) "Carrier" with periodical symptoms; (iii) Amoebic colitis; (iv) Amoebic dysentery.

The cultures, grown on BOECK and DRBOHLAV's diphasic medium, contained vegetative forms and cysts. The cysts were measured on every culture.

As according to several authors the young cat is the animal most sensitive to infection with *E. histolytica*, kittens were used in the experiments; they were laparotomized and infected intracoecally with 3 ml of the culture's sediment.

The experiments were evaluated histologically. After sacrificing the animals the intestines, heart, kidneys, liver and spleen were fixed in formalin, and the sections were stained with heamatoxylin and eosin.

Results

The results are summarized in Table I.

Table I

Data showing infection of the animals and the clinical course of the disease

Designation of animal	Classification of patient's condition according to CRAIG	Size of cysts	Time of		Faecal examination	Clinical course
			inoculation	death		
M. 1./1958.	II.	13 μ	June 11, 1958	Oct. 30, 1958	Periodically positive	Symptomless for 4 months; then severe
M. 2./1958.	IV.	15 μ	June 4, 1958	Nov. 4, 1958	Positive on Oct. 26	Symptomless for 3½ months; then severe
M. 3./1958.	II.	23 and 13 μ	June 11, 1958	Nov. 4, 1958	Negative throughout	Symptomless for 4 months; then severe
M. 4./1958.	III.	19 and 23 μ	Nov 21, 1958	Dec. 24, 1958	Not done	Died
M. 5./1959.	III.	16 and 18 μ	June 19, 1958	Feb. 25, 1959	Negative throughout	Symptomless for 5 months, then severe. Sacrificed

As seen in Table I, there was no correlation between the size of cysts and the clinical course of the disease either in patients or in animals. Gross and histological examinations also confirmed this observation.

All the animals died with clinical symptoms of dysentery and colitis with the exception of cat M. 5, which was killed. Until symptoms had developed, which occurred generally 3 or 4 months after the intracoeccal infection, the condition of the animals seemed to be more or less normal. In cat M. 1 which was excreting *Trichomonas* with the faeces when the inoculation was done, the ensuing disease was clinically more severe than in the other animals. The faeces of the cats were regularly examined. Cats M. 1 and M. 2, which displayed the gravest symptoms, regularly excreted *E. histolytica*. Because of

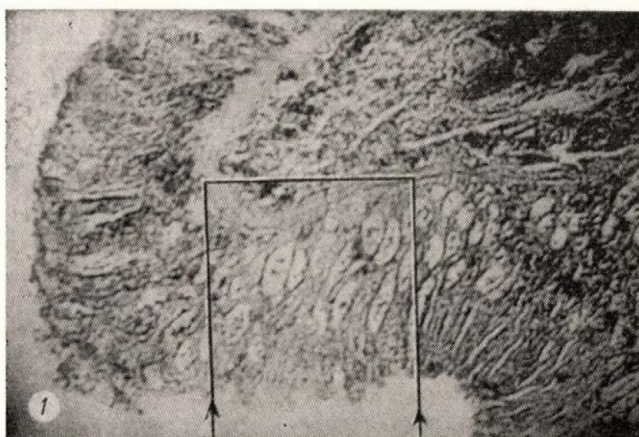


Fig. 1. Colon. Partial necrosis of mucosa (framed area). Magnification about $\times 40$

its early death, no faecal samples of animal M. 4 were examined. *E. histolytica* could not be isolated from cats M. 3 and M. 5 during the whole period of examination.

Post mortem all the five animals showed fairly identical changes. The mucosa of the colon was thickened with elevated lymphoid plaques. In the colon of the animals with gravest symptoms, haemorrhages were visible in the submucosa. In the lower part of the small intestine aspecific inflammatory changes accompanied with oedematous swelling of the whole mucosa and hypertrophy of the lymphoid plaques were seen, and the intestinal serosa was covered with thin fibrinous deposits.

The spleen was enlarged, hyperplastic in all the animals. Other organs, as the heart and kidney, showed non-specific changes usual with chronic infections (parenchymatous degeneration, etc.). Liver abscesses were not discovered in the animals.

The sections showed the most severe changes in the colon.

The mucosa of the colon was swollen, the intestinal glands contained numerous *E. histolytica*, especially in animals M. 1 and M. 2. In cat M. 2 part

of the mucosa was necrosed, but no ulcer was demonstrable. Under high power the amoebae are clearly visible (see *Figs. 2 and 3*).

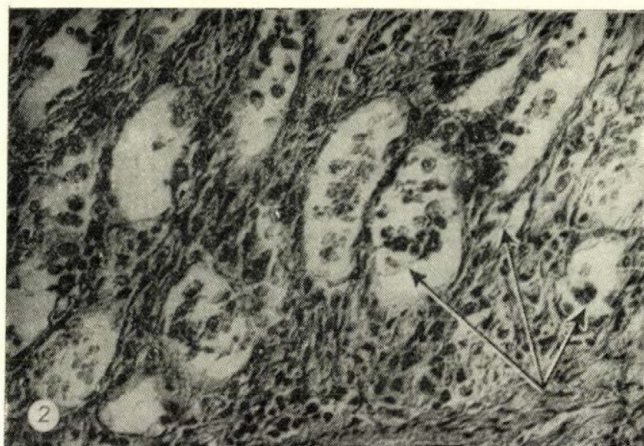


Fig. 2. Colon. Partial necrosis of mucosa. Large numbers of *E. histolytica* in the lumen of the intestinal glands. Magnification about $\times 120$

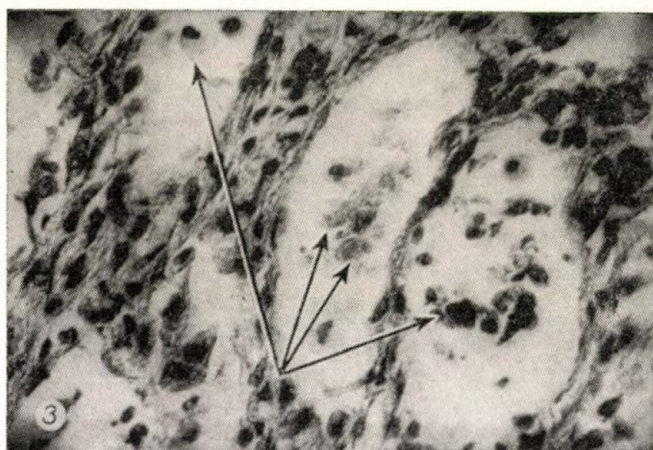


Fig. 3. Colon. Partial necrosis of mucosa. Magnification about $\times 300$

Apart from an oedematous swelling and round cell infiltration, no specific changes appeared in the submucosa.

In cases M. 3, M. 4 and M. 5, the inflammatory changes were milder. In the mucous glands of the colon a small number of *E. histolytica* was demonstrable in animal M. 3, while in M. 4 and M. 5 there were only a few amoebae.

In the small intestines a thin fibrinous deposit was seen on the serosa; all the changes were aspecific, such as inflammation of the mucosa, oedema of the submucosa and round cell infiltration.

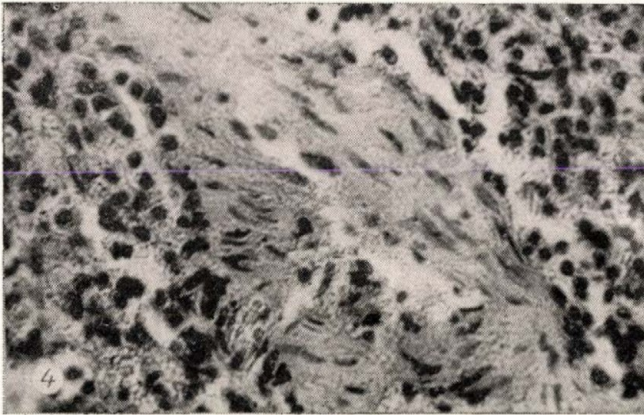


Fig. 4. Spleen. Loosening of the pulp and hypertrophy of the trabecular system. Magnification about $\times 300$

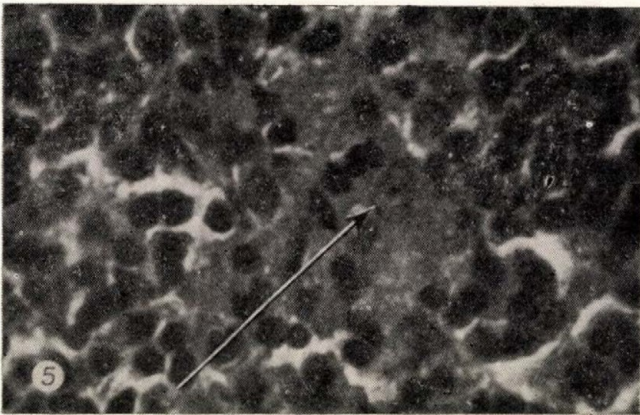


Fig. 5. Spleen. The arrow shows an *E. histolytica*. Magnification about $\times 600$

The spleen showed signs pointing to a septic condition, consisting of hypertrophy of the connective tissue trabeculae and of the malpighian corpuscles (see Fig. 4). Careful examination under high power revealed *E. histolytica* (see Fig. 5).

Discussion

According to DOFLEIN [13] *E. histolytica* is unable to multiply in the lumen of the cat's intestine. Multiplication occurs exclusively in the intestinal wall, thus only strains invading the tissues can give rise to a successful infection. It should be noted that the ability of invading the tissues means the pathogenic capacity itself.

In our experiments the intracoeccal inoculation of kittens with *E. histolytica* strains isolated from children suffering from clinically manifest amoebiasis resulted in the infected animals in chronic disease the clinical course of which as well as the *post mortem* findings were characteristic of superficial amoebiasis. In the mucous glands of the large intestine of the animals *E. histolytica* could be demonstrated. According to the severity of the disease the pathogenic agent was found in larger or smaller numbers. Inflammatory changes were present also in the milder cases. *E. histolytica* was demonstrated also in the spleen.

The findings seem to be indicative of the animal pathogenicity of *E. histolytica* strains occurring in Hungary. For final conclusions, however, further investigations are necessary.

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Address of the authors:

TIBOR BAKÁCS, MÁRIA JANKÓ

State Institute of Hygiene, Gyáli ut 2/6, Budapest IX., Hungary.

COMPARATIVE STUDY OF PSEUDOMONAS SPECIES AFFECTING HEMP AND THE MULBERRY TREE

By

Z. KLEMENT and L. LOVREKOVICH

Research Institute for Plant Protection, Budapest

(Received November 5, 1959)

Summary. *Ps. cannabina*, described in Yugoslavia in the year 1959, has been encountered in hemp-growing areas of Hungary during 1957 and 1958. The new pathogen has been found to show a close relationship to *Ps. mori* as regards biochemical features, sensitivity to phages, and antigenic structure. This resemblance is so remarkable as to make it debatable whether *Ps. cannabina* may be regarded as a new species or a forma species of *Ps. mori*.

In the years 1957—58 an unknown bacterial disease appeared in the hemp plantations near the Yugoslav border of Hungary [4]. Judged by its properties, the pathogen isolated from infected plants showed the closest resemblance to *Pseudomonas mori* (Boyer and Lambert) Stevens which in the mulberry tree causes spots on the leaves and withering of shoots. Therefore we thought that the pathogen of hemp may be either identical with *Ps. mori* or a hitherto undetected variant of the bacteria belonging to this group. This assumption induced us to undertake a comparative inquiry into the bacterial strains of hemp and the mulberry tree. These investigations involved comparisons between the strains isolated from mulberry trees and those from hemp regarding their biochemical and pathological properties, sensitivity to phages, as well as their antigenic structure.

In the meantime, a new pathogen of hemp was described in Yugoslavia in 1959 which has been given the name of *Ps. cannabina* by ŠUTIĆ and DOWSON [6]. Our studies revealed the identity of this pathogen with the bacterium isolated in Hungary. Since ŠUTIĆ and DOWSON did not study the relationship between *Ps. cannabina* and *Ps. mori*, the pathogen of a host plants belonging to the same family, we thought that our pertaining results might be interesting.

Materials and methods

The bacterial strains were isolated on broth agar. Biochemical investigations were made with the usual methods [3]. Carbohydrate breakdown was observed for twenty-five days in peptone-free synthetic media containing 1 per cent sugar or sugar-like alcohol, in the presence of Andrade's indicator.

Phage studies were carried out in broth agar, by the agar-layer method. The phages were obtained from heavily infected plant tissue. The plant tissues were homogenized and suspended in tenfold volumes of broth and this was inoculated with the indicator bacterium. After 24 to 28 hours' incubation the suspension was centrifuged and the supernatant passed through a filtre retaining bacteria. From the agar plates containing indicator bacteria prepa-

red with the filtrate, the plaques were isolated and their purity ascertained. The cultures were kept at 28° C.

For serological investigations the sera were prepared as follows. The bacteria grown in 24 hours on the broth agar were washed off with physiological saline. Prior to inoculation into rabbits, the suspensions were boiled for two hours in a water-bath. Immunization was performed by injecting intravenously every other day 1, 2, 5, 5 ml doses from suspensions of Brown's No. 4 concentration. The animals were bled seven days after the last inoculation. Before evaluation, the agglutination tubes were first kept for two hours in a water-bath of 37° C, and then left standing at room temperature overnight.

Suspensions prepared with physiological saline of 24 to 48 hours' fresh agar cultures were used for the infection of plants. The suspensions were sprayed on the leaves. After infection, the plants were kept in a humid atmosphere for three days.

Results

Morphology and biochemical properties. Several bacterial strains were isolated from affected hemp. At the same time a number of bacterial strains were isolated from infected mulberry shoots collected in other parts of the country. As reported in another paper [5], the strains isolated from mulberry trees fell into two groups as regards biochemical properties, sensitivity to phages, and antigenic structure. The strains constituting group *A* corresponded to *Ps. mori* as described by DOWSON [2]. The strains in group *B* were regarded as a new variant which we designated *Ps. mori*-var. *huszi* [5].

Colonies of strains from hemp and mulberry trees showed the same morphology. Hemp bacteria differed from mulberry bacteria only by slower growth, and by the diffusion of a caramel coloured dye into the culture medium.

Table I summarizes the biochemical properties of the strains.

Bacteria from hemp and mulberry differed mainly in nitrate reduction and slightly in raffinose and mannitol breakdown. Hemp bacteria did not produce acid from these sugars, whereas slight acid formation was induced by *Ps. mori* strains after ten days. The pathogen of hemp was found to stand biochemically nearer to *Ps. mori* than to *Ps. mori* var. *huszi*.

Pathology. *Cannabis sativa* plants and young *Morus alba* trees were infected in a greenhouse with cultures of *Ps. cannabina*, *Ps. mori*, and *Ps. mori* var. *huszi*. With *Ps. cannabina* strains infection was achieved only on hemp, whereas, with strains of *Ps. mori* and *Ps. mori* var. *huszi* only on mulberry leaves and shoots. The experiments apart from furnishing evidence confirming the pathogenicity of the strains revealed differences in the pathogenicity of *Ps. cannabina*, *Ps. mori* and *Ps. mori* var. *huszi*. It should be remembered that hemp and the mulberry tree belong to the same family.

Phage sensitivity. Two bacteriophages were isolated from heavily infected hemp. The variety inducing smaller, opaque plaques, 2 to 3 mm in diameter, with well-defined margins, was designated CaP₁ (Fig. 1a); the phage eliciting larger clear plaques, 5 to 15 mm in diameter, with definite margins was marked CaP₂ (Fig. 1b). The latter plaques were surrounded with a pale

Table I
Biochemical properties of hemp and mulberry strains

	<i>Ps. cannabina</i>	<i>Ps. mori</i>	<i>Ps. mori</i> var. <i>huszi</i>
Gelatine liquefaction	—	—	—
Starch hydrolysis	—	—	+
Hydrogen sulphide formation....	—	—	—
Ammonia formation.....	+	+	+
Nitrate reduction	—	+	+
Indole formation.....	—	—	—
Milk { coagulation.....	—	—	—
peptonization	—	—	—
alkalization	+	+	+
Carbohydrate breakdown:			
1. arabinose	++	++	+—
2. xylose.....	++	++	+
3. glucose	++	++	+—
4. fructose	++	+	+
5. mannose	++	++	+—
6. galactose	++	++	+—
7. saccharose	+—	+	+—
8. maltose	—	—	—
9. lactose	—	—	—
10. raffinose.....	—	+	+—
11. dextrin	—	—	—
12. dulcitol	—	—	—
13. glycerin	+—	+—	+—
14. salicin.....	—	—	—
15. mannitol	—	+—	+—

Carbohydrate breakdown: ++ marked, + moderate, +— faint formation of acid

halo after 48 hours. Phage CaP₁ lysed not only hemp strains but also *Ps. mori* and *Ps. mori* var. *huszi*, while phage CaP₂ acted only on *Ps. mori* var. *huszi* in addition to hemp strains.

As reported in another paper [5], infected mulberry shoots also yielded two phages, of which the variety designated MoP₁ was entirely similar to phage CaP₁. The other phage forming larger plaques (MhP₁) (Fig. 1c), though much like phage CaP₂ in plaque morphology, greatly differed from it as to its host range. The activity of the phages isolated from hemp and mulberry trees on bacteria from hemp and mulberry trees is presented in Table II. This shows

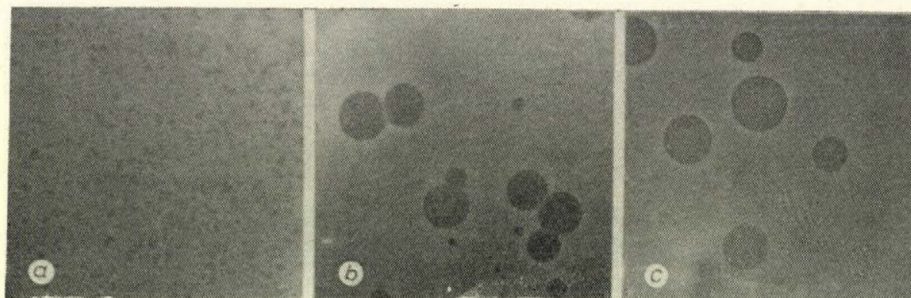


Fig. 1. Plaques of a) phage CaP₁; b) phage CaP₂; c) phage MhP₁ after incubation for 24 hour

that the phages CaP₁ and MoP₁ agreed not only in plaque morphology, but also in producing lysis of both hemp and mulberry tree strains.

Table II

Activity of phages on hemp and mulberry bacteria

Isolation from	Phage strain	Bacteria		
		<i>Ps. cannabina</i>	<i>Ps. mori</i>	<i>Ps. mori</i> var. <i>huszi</i>
Hemp	CaP ₁	+	+	+
	CaP ₂	+	—	+
Mulberry tree	MoP ₁	+	+	+
	MhP ₁	—	—	+

To determine their host range, the phages were tested for their action on other phytopathogenic species of *Pseudomonas*, as shown in Table III. The activity spectrum of the two phages was identical. This supports the supposition that phages CaP₁ and MoP₁ were identical.

The identity of phages CaP₁ and MoP₁ was also proved by their cross resistance. The test involved separate infection of all hemp and mulberry strains with massive amounts of CaP₁ and MoP₁. After the preparation of plates and incubation, phage-resistant bacterial colonies developed in the place of the plaques. The phage-resistant bacteria isolated from the colonies were cross-tested for both phages. The complete absence of bacteriolysis was another proof of the identity of the two bacteriophages, a remarkable fact considering their having originated from different parts of the country.

Phage CaP₂ also proved to be polyvalent, inasmuch as it acted on three different species of *Pseudomonas* in addition to homologous hemp strains and *Ps. mori* var. *huszi*.

The CaP₁ bacteriophage isolated from hemp were found to cause lysis of mulberry bacteria, a fact pointing to the close relationship between hemp and mulberry bacteria.

Serology. A comparison of the antigenic structure of the strains promised to yield interesting results. For agglutination tests, sera from *Ps. cannabina* strain LO2, *Ps. mori* strain TO1, and *Ps. mori* var. *huszi* strain TO6 were used. The results are presented in Table IV. As *Ps. mori* var. *huszi* serum failed to agglutinate strains of *Ps. cannabina*, and *Ps. cannabina* serum failed to agglutinate *Ps. mori* var. *huszi* strains, these tests are not shown in Table IV. As can be seen, strains of *Ps. cannabina* and *Ps. mori* showed a similar behaviour in the agglutination tests.

Table III

Phage sensitivity and serum titres of phytopathogenic Pseudomonas species

Species of <i>Pseudomonas</i>	Strain	Phage			Serum titre	
		MoP ₁	CaP ₁	CaP ₂	<i>Ps. cannabina</i> (LO2)	<i>Ps. mori</i> (TO1)
<i>Ps. aeruginosa</i>	288	—	—	—	—	—
<i>angulata</i>	263	—	—	—	—	—
<i>atrofaciens</i>	117	—	—	—	—	—
<i>cannabina</i>	LO2	+	+	+	1280	1280
<i>caryophylli</i>	349	—	—	—	—	—
<i>glycinea</i>	286	+	+	—	—	—
<i>lachrymans</i>	RO16	+	+	—	—	80
<i>marginalis</i>	MO56	—	—	+	—	—
<i>mellea</i>	280	—	—	—	—	1280
<i>mori</i>	TO1	+	+	—	640	640
<i>mori</i> var. <i>huszi</i>	TO6	+	+	+	—	40
<i>mors-prunorum</i>	330	+	+	—	80	80
<i>oryzicola</i>	A309	—	—	—	—	—
<i>medicaginis</i> f. sp. <i>phaseolicola</i> .	El356	+	+	—	—	80
<i>pisi</i>	PO18	—	—	—	—	—
<i>syringae</i>	(Volcani)	—	—	+	320	160
<i>syringae</i> var. <i>capsici</i>	I201	+	+	—	640	160
<i>syringae</i> f. sp. <i>populea</i>	294	+	+	—	—	—
<i>tabaci</i>	HO49	—	—	—	—	40
<i>tomato</i>	269	—	—	+	640	640

The strains designated only with figures have been obtained from the *National Collection of Plant Pathogenic Bacteria, England*; those marked with letters have been isolated by ourselves

With a view to a close investigation of the antigenic structure, cross exhaustion tests were performed; these disclosed a close resemblance, but not complete, identity of the antigenic structure of *Ps. cannabina* and *Ps. mori*.

Table IV

Titre of Ps. cannabina (LO2) serum with strains of Ps. cannabina and Ps. mori

Antigen	Serum dilution 1 :										
	10	20	40	80	160	320	640	1280	2560	5120	Control
<i>Ps. cannabina</i>	LO1	+++	+++	+++	+++	+++	+++	+++	+++	++	—
	LO2	+++	+++	+++	+++	+++	+++	+++	+++	++	—
<i>Ps. mori</i>	TO1	+++	+++	+++	+++	+++	+++	—	—	—	—
	TO5	+++	+++	+++	+++	+++	+++	++	—	—	—

Titre of Ps. mori (TO1) serum with strains of Ps. cannabina and Ps. mori

<i>Ps. cannabina</i>	LO1	+++	+++	+++	+++	+++	+++	+++	+++	++	—
	LO2	+++	+++	+++	+++	+++	+++	+++	+++	++	—
<i>Ps. mori</i>	TO1	+++	+++	+++	+++	+++	+++	—	—	—	—
	TO5	+++	+++	+++	+++	+++	+++	++	—	—	—

Next we examined the agglutination of other phytopathogenic *Pseudomonas* species. The results of these investigations are presented in Table III. Although fewer species were agglutinated by *Ps. cannabina* serum than by that of *Ps. mori*, all the species agglutinated by *Ps. cannabina* serum, reacted also to *Ps. mori* serum.

The serological investigations yielded further evidence of the slight difference in antigenic structure between the two bacteria.

Discussion

When our pathogenic strains were isolated from hemp, the pathogen of hemp was unknown in the literature. The numerous common properties of the pathogen of hemp and the bacteria of the mulberry tree have led us to suppose that the hemp bacterium is either identical with *Ps. mori* or one of its forma species. Pathological investigations have disproved the first assumption.

There are several examples where two phytopathogenic bacteria manifesting biochemical similarity differ pathologically; these are forma species.

An illustrative instance is furnished by *Ps. medicaginis* and *Ps. medicaginis* f. sp. *phaseolicola* [3] where BURKHOLDER [1] could establish only pathological differences between the two bacteria. While *Ps. medicaginis* affects only lucerne, and *Ps. medicaginis* f. sp. *phaseolicola* only beans, biochemically they display a close relationship. Having discovered the presence of a similar biochemical resemblance between *Ps. cannabina* — described in the meantime — and *Ps. mori*, we supposed the former to be only a variant of the latter. Since pathological and biochemical investigations failed to produce a conclusive answer to the question whether the pathogen isolated from hemp should be classified as a new species or as a forma species of *Ps. mori*, our studies had to be extended to phages and serological investigations. These experiments revealed the existence of a very near relationship between the hemp bacterium and *Ps. mori*. This relationship is so close as to make it questionable whether *Ps. cannabina* should be regarded as a new species or only a forma species of *Ps. mori*. As long as the concept of a species among the phytopathogenic *Pseudomonas* variants lacks a final definition, we regard the pathogen of hemp termed *Ps. cannabina* by ŠUTIĆ and DOWSON as a species closely related to *Ps. mori*.

Our investigations have also shown that in biochemical properties and antigenic structure *Ps. cannabina* displays a more marked resemblance to *Ps. mori* than to *Ps. mori* var. *huszi*.

Judging by pigment formation ŠUTIĆ and DOWSON claimed *Ps. cannabina* to stand near to *Ps. caryophylli*. Our phage experiments and serological findings did not confirm the alleged relationship between *Ps. cannabina* and *Ps. caryophylli*. It must, however, be mentioned that our *Ps. caryophylli* strain obtained from the *National Collection of Plant Pathogenic Bacteria in England* did not produce any caramel coloration.

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Address of the authors:

ZOLTÁN KLEMENT
LÁSZLÓ LOVREKOVICH

Research Institute for Plant Protection, Herman Ottó út 15, Budapest II., Hungary

TWO-LAYER POLYTROPHIC CULTURE MEDIUM (CIG) FOR SIMULTANEOUS INVESTIGATION OF CITRATE UTILIZATION, INDOLE FORMATION AND GELATINE LIQUEFACTION

By

M. RODLER, S. VÖRÖS and P. HEGYI

Institute of Microbiology, University Medical School, Pécs

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Summary. The citrate-indole-gelatine (CIG) culture medium has been found to allow reliable demonstration of the utilization of citrate, formation of indole, and liquefaction of gelatine by intestinal bacteria. Applied together with NÓGRÁDY—RODLER's polytrophic culture medium, it ensures prompt and correct biochemical diagnosis of intestinal bacteria.

The polytrophic culture medium of NÓGRÁDY—RODLER, greatly facilitating the differentiation of intestinal bacteria, makes it possible to determine some fundamentally important biochemical properties [5, 6]. However, reliable identification calls for the evidence of further biochemical reactions. Of these, the production of indole and gelatinase and the demonstration of citrate utilization are the most important.

Many attempts have been made at evolving a culture medium capable of providing rapid information about various biochemical properties [17, 18].

The combined demonstration of fermentation yielding one or several carbohydrates (lactose, saccharose, dextrose, perhaps even mannite), of hydrogen sulphide development, urea breakdown, or indole production have been improved by polytrophic culture media [1—6, 9—12, 15, 16].

It is a difficult task to demonstrate indole formation in a culture medium incorporating carbohydrate [8, 11, 13]. Though RAPPAPORT found that indole production was not affected by low concentrations of mannite or laevulose [11], by their use we are, nevertheless, deprived of the known favourable carbohydrate composition, since in the presence of mannite, for instance, no information is obtained about the fermentation of mannite-negative *Shigella*. Subsequent addition of lactose solution in doubtful cases and fresh incubation involve wearisome, lengthy procedures.

The above considerations suggested to separate the demonstration of indole formation from the investigation of carbohydrate fermentation in our new polytrophic culture medium.

In the past fifty years the methods for indicating indole formation have been discussed by many authors [13]. A great number of workers employ the so-called paper strip method, as for instance BRAUN and SILBERSTEIN [7], SILBERSTEIN and RABINOVITZ [7a], RAPPAPORT *et al.* [11]. This method, however, is not fully reliable, particularly with slight indole formation, and is

practically useless for demonstration of the non-volatile alphanaphthyl indole [8, 13]. ISENBERG and SUNDHEIM have compared the methods in general use; they have questioned the reliability of direct p-dimethyl-amidobenzaldehyde tests and recommended the use of a new reaction [19]. We ourselves have discontinued use of the paper strip method and instead are adding KOVÁCS' or EHRlich-BÖHME's reagent to the cultures.

As concerns the test for gelatine liquefaction, three methods are being generally applied, *viz.* gelatine culture medium [8]; FeCl_2 gelatine culture medium, which permits at the same time determination of H_2S production [14]; agar plate containing gelatine which with HgCl_2 dissolved in HCl as the reagent offers special advantages in the study of late or weak gelatine liquefactors [13]. The use of simple gelatine culture media has been adapted by us to exclude rapid and intensive gelatine liquefactors.

In the investigation of citrate utilization, the KOSER's and SIMMONS' citrate culture media generally employed [8, 13, 14] are well suited for the demonstration of sodium and ammonium citrate utilization. Hence we did not modify SIMMONS' citrate agar and are using it in combination with our indole gelatine culture medium.

Materials and methods

Some preliminary experiments furnished evidence that the study of indole formation and the liquefaction of gelatine may proceed simultaneously. It was furthermore ascertained that the use of a two-layer culture medium makes undisturbed study of citrate utilization possible beside demonstration of the above-mentioned reactions. By the preparation of a two-layer culture medium permitting the study of three additional biochemical signs a culture medium was obtained which virtually completed NÓGRÁDY-RODLER's polytrophic culture medium and rendered biochemical determination faster and more conclusive.

Media. The culture medium suitable for the concurrent investigation of citrate utilization, indole formation, and the liquefaction of gelatine, consists of two parts, *viz.* I. Simmons' citrate agar; II. Indole culture medium.

I. *Simmons' citrate agar*: Magnesium sulphate 0.2 g, monoammonium phosphate 1.0 g, dipotassium phosphate 1.0 g, sodium citrate 2.0 g, bromthymol blue 0.08 g (8.0 ml of an alcoholic 1 per cent solution of bromthymol blue), agar-agar 20.0 g, distilled water 1000 ml. The above ratio of constituents provides for the required pH (6.8).

II. *Indole-gelatine culture medium*: 1. Peptone Bacto 20.0 g, gelatine 120.0 g, sodium chloride 6.0 g, tryptophan 0.025 g, distilled water 1000.0 ml, pH 6.8 to 7.0 (correction with about 20 ml of N NaOH).

2. Peptone Richter 150.0 g, gelatine 120.0 g, sodium chloride 5.0 g, distilled water 1000.0 ml, pH 6.8 to 7.0 (correction with about 20 ml of N NaOH).

The constituents of culture medium I are dissolved by boiling in 1000 ml of distilled water; the solution is filtered through cotton wool, and transferred into 5 ml test tubes or Widal-tubes in amounts of 2.5 ml; after sterilization in flowing steam slant cultures are made.

The constituents of culture medium II are dissolved by boiling in 1000 ml of distilled water. Filtration is performed through cotton wool, pH is corrected to 6.8 to 7.0 with Na OH . After sterilization in flowing steam, the solution is distributed under aseptic conditions as corner parts in quantities of 2.0 to 0.5 ml paying attention lest the gelatine medium flow along the surface of the slant Simmons' agar. In a refrigerator it will keep three to four weeks without any loss of value.

Reagents for the indole test: Kovács' reagent: p-dimethyl amidobenzaldehyde 10 g, amyl alcohol 150 g, concentrated HCl 50 g, or

Ehrlich-Böhme's reagent: p-dimethyl amidobenzaldehyde 8.0 g, 95 to 96 per cent ethyl alcohol 760.0 g, pure concentrated HCl 160.0 ml.

Inoculation of the culture medium is performed with an inoculating or platinum needle first by streaking on the slanting surface, or by linear inoculation (dispersal is started about 0.5 to 1.0 cm above the gelatine medium), then by thrusting the needle into the indole-gelatine medium. Proper attention must be paid that the gelatine part of the culture medium should be solid.

Incubation : at 37° for 24 to 48 hours (sometimes longer).

Evaluation : results are read in the following order: 1. Utilization of citrate; 2. Liquefaction of gelatine; 3. Indole production.

Adherence to this order is essential, because by addition of the acid indole reagent (Kovács, Ehrlich-Böhme) the culture medium is made acid, rendering the registration of pH changes impossible. Utilization of citrate is shown by growth and the change of colour (green to blue) following alkalescence which as a rule becomes discernible in eighteen hours; incubation for forty-eight hours is nevertheless believed to be indispensable for the demonstration of later utilization.

Before the evaluation of gelatine liquefaction, the culture medium is cooled below the congealment temperature of gelatine, at +4 °C in a refrigerator (or cold water-bath) for 60 minutes. Reading of gelatine liquefaction should take place after forty-eight hours' incubation.

Indole formation is indicated by the appearance of a red colour in the gelatine upon addition of some drops of Kovács' or Ehrlich-Böhme's reagent; the reaction takes about five minutes to develop. It is expedient to add the reagent (about 5 to 10 drops) to the dissolved indole-gelatine culture medium kept previously in a thermostat. The duration of incubation should be forty-eight hours also for the indole test.

Table I

Behaviour of various intestinal bacteria on polytrophic culture media I (N-R) and II (CIG)

	Polytrophic I (N-R)				Polytrophic II (CIG)		
	Ureum	H ₂ S	Dextrose with or without gas	Lactose	Citrate	Indole	Gelatine
Salmonella	—	+	++	—	+	—	— (.+)
<i>S. typhi</i>	—	+	+-	—	v	—	—
Arizona	—	+	++	v	+	—	v
Ballerup } Bethesda } <i>E. freundii</i> }	— (+)	+	++	v	+	—	—
<i>Citrobacter</i>							
<i>E. coli</i>							
Klebsiella	+	—	++	+	+	—	—
Cloaca	v	—	++	v	+	—	v
Hafnia	—	(+)	++	—	v	—	—
Shigella	—	—	+-	—	—	v	—
<i>Proteus vulgaris</i>	+	+	++	—	v	+	+
<i>Proteus mirabilis</i>	+	+	++	—	v	—	+
<i>Proteus morganii</i>	+	—	++	—	—	+	—
<i>Proteus rettgeri</i>	+	—	v	—	+	+	—
Providencia	—	—	v	—	+	+	—

Signs : + = positive reaction
 — = negative reaction
 (+) = rarely or slightly positive
 v = varying
 ++ = acid + gas
 +- = acid without gas
 (.+) = some S types liquefying gelatine

By the use of Nógrády-Rodler's polytrophic medium and the citrate-indole-gelatine (CIG) medium, information may be obtained concerning the following biological properties of various intestinal bacteria: urea breakdown, H_2S and acid production, development of gas, utilization of citrate, indole formation and gelatine liquefaction; with the help of these characteristics, successful biochemical differentiation becomes feasible (see *Table I*).[†]

Results

Before proceeding to the investigation of a larger number of strains from our routine material, we thought it necessary to study the behaviour of standard strains from our collection in parallel on polytrophic culture medium I (Nógrády—Rodler), and polytrophic culture medium II (CIG). The results have been summed up in *Table II*.

The 100 *Salmonella* strains under observation — including four strains of *S. typhi* — displayed the following biological properties. Hydrolysis of urea was not observed in any case; production of H_2S and utilization of citrate was demonstrated with 98 strains, while one strain (*S. abortus bovis*) proved to be liquefacient. Simultaneous development of acid and gas occurred with 96 strains. One strain each from the Arizona, Ballerup, and Bethesda groups manifested the properties characteristic of these groups.

Each of the 50 standard *E. coli* "O" strains conformed to the characteristics. Of the first 30 "K" type *Klebsiella* strains urea was hydrolysed by 29 (the exception was type K 3); citrate was utilized by 28 (types K 1 and K 3 formed exceptions); development of H_2S , liquefaction of gelatine, and indole formation were not observed with any of the strains.

The 50 *Shigella* strains from our collection — belonging mainly to the Flexner group — gave the following results: hydrolysis of urea, production of H_2S , utilization of citrate, liquefaction of gelatine were not displayed by any of the strains, while production of gas was observed with one, and indole formation with 5 strains.

Of our standard *Proteus Hauseri* strains all formed H_2S and decomposed urea; 4 strains did not liquefy gelatine after forty-eight hours' incubation; the indole test was positive in 19 cases, negative in 31, a fact conclusive of the biochemical difference between *P. vulgaris* and *P. mirabilis*, in full agreement with data published in the pertaining literature [14].

Our *P.morganii* strains — collected for another study — showed similar behaviour on CIG medium and upon detailed biochemical investigation [20].

Of 20 *Providencia* strains* 3 decomposed urea; none formed H_2S , none liquified gelatine; 19 formed indole and utilized citrate. *Hafnia* 118, *Cloaca cloacae* 6279, as well as the only *Serratia* strain examined showed characteristic biochemical properties.

* We are indebted to Dr. W. H. EWING, *Chemlee, U. S. A.*, for making available these strains.

Table II
Behaviour of subcultures of standard intestinal bacteria on polytrophic culture media I and II

	No	Polytrophic I (N—R)								Polytrophic II (CIG)						Remark
		Ureum		H ₂ S		Dextrose				Citrate		Indole		Gelatine		
						Acid		Gas								
		+	—	+	—	+	—	+	—	+	—	+	—	+	—	
Salmonella	100	0	100	98	2	100	0	96	4	98	2	0	100	1	99	<i>P. vulg.</i> 19 <i>P. mirab.</i> 31 late lique- faction
Arizona orig.	1	0	1	1	0	1	0	1	0	1	0	0	1	1	0	
Ballerup orig. 107	1	0	1	1	0	1	0	1	0	1	0	0	1	0	1	
Bethesda Na la	1	0	1	1	0	1	0	1	0	1	0	0	1	0	1	
<i>E. coli</i> (stand. “O”)	50	0	50	0	50	50	0	50	0	0	50	50	0	0	50	
Klebsiella (stand “K”)	30	29	1	0	30	30	0	30	0	28	2	0	30	0	30	
<i>C. cloacae</i> 6279	1	0	1	0	1	1	0	1	0	1	0	0	1	1	0	
Hafnia 118.....	1	0	1	0	1	1	0	0	1	0	1	0	1	0	1	
Shigella (collection)	50	—	50	0	50	50	0	1	49	0	50	5	45	0	50	
<i>P. hauseri</i> (stand. “O” and “H”)	50	50	0	50	0	50	0	0	50	12	38	19	31	46	4	
<i>P. morgani</i>	30	30	0	0	30	30	0	0	30	2	28	30	0	0	30	
Providencia	20	3	17	0	20	20	0	0	20	19	1	19	1	0	20	

Table III

Behaviour of freshly isolated strains of intestinal bacteria on polytrophic culture media I and II

	No	Polytrophic I (N—R)								Polytrophic II (CIG)						Remark
		Ureum		H ₂ S		Dextrose				Citrate		Indole		Gelatine		
						Acid		Gas								
		+	—	+	—	+	—	+	—	+	—	+	—	+	—	
<i>E. coli</i>	506	0	506	0	506	506	0	506	0	12	494	493	13	0	506	* “Usually no indole formation” Bergey’s M. 7th edition, page 99. Probably “false” reaction. Zinsser’s Textbook. 10th edition, page 488.
<i>Citrobacter</i>	8	0	8	8	0	8	0	8	0	6	2	0	8	0	8	
<i>Klebsiella</i>	192	192	0	0	192	192	0	192	0	176	16	22	170	0	192	
<i>Alcalescens-Dispar</i>	2	0	2	0	2	2	0	0	2	0	2	2	0	0	2	
<i>Sh. flexneri</i>	31	0	31	0	31	31	0	0	31	0	31	13	18	0	31	
<i>Sh. sonnei</i>	9	0	9	0	9	9	0	0	9	0	9	0	9	0	9	
<i>C. cloacae</i>	1	0	1	0	1	1	0	0	1	1	0	0	1	1	0	
<i>Providencia</i>	6	0	6	6	0	6	0	0	6	1	5	0	6	0	6	
<i>S. typhi</i>	6	0	6	6	0	6	0	0	6	4	2	0	6	0	6	
<i>Salmonella</i>	16	0	16	16	0	16	0	16	0	16	0	0	16	0	16	
<i>Ps. pyocyanea</i>	29	—	—	—	—	—	—	—	—	26	3	24*	5	29	0	
<i>Proteus</i>	312	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
<i>P. vulgaris</i>	50	50	0	50	0	50	50	0	50	43	7	50	0	50	0	
<i>P. mirabilis</i>	176	176	0	176	0	176	0	0	176	149	27	0	176	175	1	
<i>P. morganii</i>	78	78	0	0	78	78	0	0	78	3	75	78	0	0	78	
<i>P. rettgeri</i>	7	7	0	0	7	7	0	5	4	7	0	7	0	0	7	
<i>Serratia</i>	4	—	—	—	—	—	—	—	—	4	—	0	4	4	0	
<i>Others</i>	10	0	10	0	10	10	0	0	10	7	3	0	10	0	10	
Undefinable by Polytrophic Media I and II.																

Thereafter 1122 strains of various intestinal bacteria isolated from our routine material were studied on polytrophic culture media I and II (CIG). The results are presented in *Table III*.

On the basis of their biochemical characteristics, the investigated strains showed the following distribution: *E. coli*, 506 strains; *Klebsiella*, 192; *Proteus*, 312; *Proteus vulgaris*, 50; *P. morganii*, 78; *P. mirabilis*, 176; *P. rettgeri*, 7; *Citrobacter*, 4; *Pseudomonas*, 29; *Salmonella*, 16; *S. typhi*, 6; *Sh. flexneri*, 31; *Sh. sonnei*, 9; *Providencia*, 6; *Serratia*, 4; *Cloaca cloacae*, 1; *Alcalescens Dispar*, 2; other strains (defying differentiation by polytrophic culture media I and II), 10.

In the case of late or weak positivity, or of incongruous behaviour, the reaction should be performed separately by resorting to some classical method. Our atypically behaving strains were controlled by the most appropriate classical method, and no difference was demonstrated between the results obtained, as shown in *Table IV*.

Table IV

Comparative study of freshly isolated strains of intestinal bacteria on polytrophic culture medium II and by classical methods

	No	Polytrophic II (CIG)						Simmons		Peptone water (Kovács' reagent)		Gelatine Agar-plates Clarke's reaction	
		Citrate		Indole		Gelatine		Citrate		Indole		Gelatine	
		+	—	+	—	+	—	+	—	+	—	+	—
<i>E. coli</i>	52	4	48	48	4	0	52	5	47	49	3	0	52
<i>Klebsiella</i>	53	49	4	6	47	0	53	47	6	6	47	0	53
<i>Proteus</i>	49	—	—	—	—	—	—	—	—	—	—	—	—
<i>Proteus vulgaris</i>	3	2	1	3	0	3	0	2	1	3	0	3	0
<i>Proteus mirabilis</i>	46	44	2	0	46	46	0	42	4	0	46	46	0

The use of the two polytrophic media, thus allows identification within 48 hours. A biochemically well-defined strain of intestinal bacteria can be determined serologically with ease; the amount of bacteria required for slide agglutination is readily available from polytrophic culture medium I.

In order to avoid eventual errors, the Voges-Proskauer and methyl red reactions should be performed simultaneously. They can be carried out easily by the use of one culture medium (dextrose-peptone water).

Discussion

In order to ensure prompt and correct biochemical determination, we believe it is necessary to use at least two combined culture media by whose aid the characteristic properties of intestinal bacteria can be demonstrated reliably.

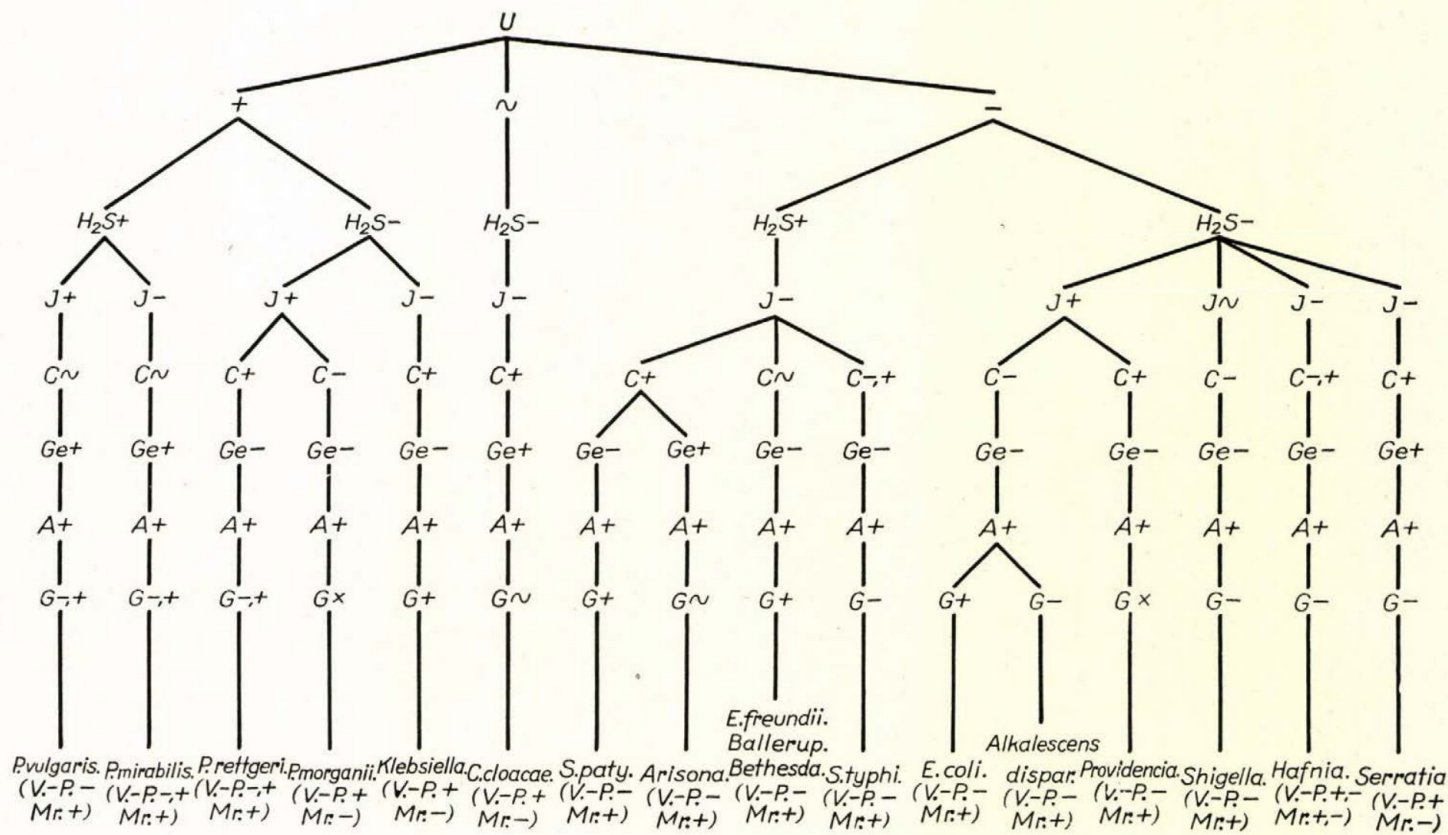


Fig. 1. Differentiation of intestinal bacteria by the use of the polytrophic media I and II (CIG)

Signs: U = urea
I = indole
C = citrate
Ge = gelatine

A = acid
G = gas
V.-P. = Voges-Proskauer's reaction
Mr. = methyl red reaction

+ = positive
- = negative
v = varying
x = late or weakly positive

Pathogenic and apathogenic intestinal bacteria isolated from Endo, DC, eosin methylene blue, bisulphite, or brilliant green agar, display characteristic growth on polytrophic culture media I and II, from which their biochemical identity is easily established (see *Table I*). Polytrophic culture medium I supplies information concerning H_2S production, urea decomposition, carbohydrate fermentation; polytrophic culture medium II concerning citrate utilization, gelatine liquefaction, and indole formation. On the basis of these biochemical characteristics, the groups of intestinal bacteria can be clearly differentiated, as demonstrated in our schematic figure (see *Fig. 1*).

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Address of the authors:

MIKLÓS RODLER

Public Health Laboratory, Vörösmarthy u. 5, Szekszárd, Hungary

SÁNDOR VÖRÖS, PÁL HEGYI

Institute of Microbiology, University Medical School, Rákóczi ut 2, Pécs, Hungary

STUDIES ON THE VARIATION IN THE ANTIGENIC STRUCTURE OF INFLUENZA A-2 VIRUS*

By

GY. TAKÁTSY and KATALIN BARB

State Institute of Hygiene, Budapest

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Summary. Influenza A-2 strains isolated in Hungary in the years 1957, 1958 and 1959 were investigated for antigenic differences using the haemagglutination inhibition and immune serum absorption tests. It has been shown by both tests, and especially clearly by the latter, that the antigenic structure of A-2 strains, like that of A-1 strains, is submitted to a natural variation which is progressive in nature.

Since the first appearance of influenza A-2 virus Hungary has experienced 3 epidemic waves. The first one, during the pandemic, began in September and came to an end in December, 1957. Morbidity rate was unusually high, about three fourth of the population were affected. During the second wave, in February and March, 1958, the attack rate was considerably lower. Although well-defined foci were observed, the epidemic did not spread all over the country. The third wave was experienced in February and March, 1959, an exhibited an unexpectedly high morbidity rate. Virus strains were isolated from each of the three waves, and we have demonstrated an antigenic difference between the 1957 strains isolated in Hungary and the Singapore strain [1].

The experiments reported in this paper concern the antigenic differences among the Hungarian strains of A-2 virus isolated in different years, and the character of the antigenic changes; these have been compared to the progressive continuous variation of A-1 virus demonstrated in our earlier papers [2, 3, 4].

Materials and methods

Viruses. The strain influenza A-2 Singapore 1/57 was kindly supplied by the *World Influenza Centre, London*. Designation of the Hungarian strains is given in *Fig. 1*.

Isolation experiments. Eight, 11- and 13-day-old embryonated eggs were used in these experiments. Throat washings were put in CO₂ ice and then stored at -20° C until inoculated by the amniotic route, not more than two days later.

The purified concentrated viral preparations were prepared as described earlier [5].

Hyperimmune sera. These were prepared by repeated inoculations of fowls by the method published earlier [6].

Haemagglutination (HA) and HA inhibition (HAI) tests were performed in plexiglass plates using the spiral loop [7]. For cross HAI tests a working dilution was made from

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each of the hyperimmune sera to shorten the series of dilutions to be made by the spiral loop. Non-specific inhibition was negligible at the dilutions used.

Immune serum absorption tests (IAT). The immune serum to be absorbed was added to a twofold dilution series of viral preparation, and the test was performed according to the micro technique recommended by us [1]. The use of varying dilutions of virus in the IAT has two advantages. (i) The "optimum" amount of virus can be determined, *i. e.* the dose which absorbs all antibody reacting with the heterologous strain without remaining detectable as free virus in the supernatant after centrifugation; (ii) the eventual difference between the homologous and heterologous titres is demonstrated in this way in more than one IAT simultaneously.

Experimental

We succeeded in isolating A-2 strains in every phase of each of the three epidemic waves observed in Hungary. When throat washings were inoculated in parallel into embryos of different age, 13-day-old embryos were not found to be more sensitive than the younger ones. Thus, FRENCH and DINEEN's observations [8] could not be repeated with our material. When several epidemic foci were examined at the same time, nearly all the throat washings taken from some foci yielded virus while in others the isolation rate was surprisingly low or even no virus could be isolated from them.

In our previous experiments the antigenic differences among A-2 strains were demonstrated by the IAT. In the present work it was attempted to detect the differences by the less sensitive cross HAI test. The differences are expressed in term of the "d" value recommended by DÖMÖK *et al.* [9]. The higher the "d" value, the greater the antigenic difference between the two strains under study. Each square in *Fig. 1* illustrates the "d" value representing the difference between the strain on the bottom of the square's column and that at the left end of its row. The darker the square, the greater the corresponding "d" value. The order of the strains, both for columns and rows, as shown in *Fig. 1*, agrees with the chronological order of their isolation. Thus the squares near the hypotenuse of the rectangular triangle illustrate the differences between strains isolated within a relatively short period, while those near the right angle refer to pairs of strains, one of which was isolated at the beginning, the other at the end of the three-year period under study. It is clearly seen that all the squares in the bottom row are dark, *i. e.* the Singapore strain is antigenically distant from the Hungarian strains, regardless the year the latter were isolated. Cumulation of dark squares can be observed near the right angle too. The squares at the hypotenuse, on the other hand, are mostly light. The data of two strains (34/57 and 13/59) only disturb this picture. We shall attempt to explain that discrepancy in the Discussion.

The above established differences among the A-2 strains under study amply suffice for being demonstrated by the HAI test. The difference between the Singapore strain, which was isolated earlier, and the Hungarian strains is more pregnant than the differences among the Hungarian strains.

In order to show the continuity of the process of antigenic variation, thirteen A-2 hyperimmune sera were absorbed with the "optimum" dose of a strain isolated at the beginning of the 1957 epidemic, and another sample of each of the 13 sera was similarly absorbed with a strain isolated at the end

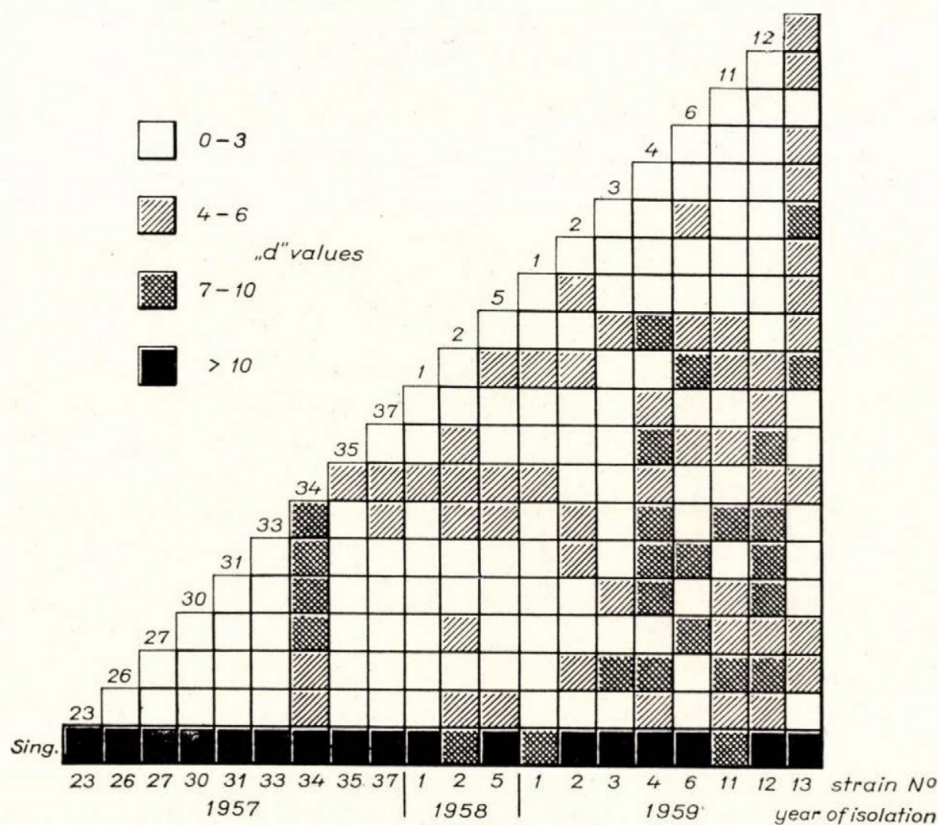


Fig. 1. The differences between some strains of A-2 influenza virus expressed in "d" values, based on the cross HAI test

of the 1959 epidemic. The sera to the strains 34/57 and 13/59 behaving irregularly in the HAI test were left out of these experiments. Fig. 2 illustrates the results.

The top of the columns illustrates the homologous HAI titres prior to absorption, the shaded columns the same titres after absorption with the "optimum" dose of the 23/57 strain (Fig. 2/a) and the 12/59 strain (Fig. 2/b), respectively. Absorption with the former strain left behind a significant amount of homologous antibody in the Singapore serum, and residual antibody was also detected in the sera which were prepared against A-2 strains isolated after the 23/57 strain. The residual titre appears to be in direct relation with

the time interval between the isolation of the two strains. The relation between interval and residual antibody was more demonstrable in the experiment illustrated in *Fig. 2/b* when the same sera were absorbed with the strain 12/59 isolated at the end of the 1959 epidemic.

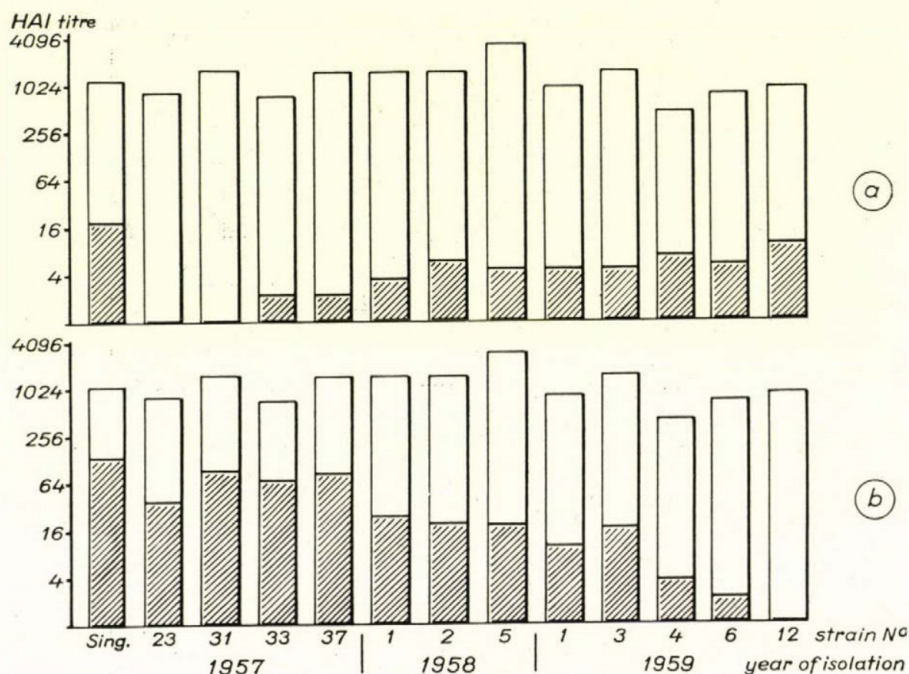


Fig. 2. Adsorption of anti-A2 hyperimmune sera with a strain isolated

(a) at the beginning of the 1957 epidemic

(b) at the end of the 1959 epidemic

Discussion

The results of the HAI tests recorded in this paper suggest that the antigenic variation of the influenza A-2 strains between October, 1957, and March, 1959, was gradual. Disregarding the two irregularly behaving strains, the longer the interval between the isolations, the greater was the difference in the antigenic structure. Nevertheless, time is not the only factor determining the degree of variation. It is supposed that minor changes in antigenic structure are induced by serial passages in partially immune persons. Thus the progress of variation is expected to depend on the number of passages (which may be different for strains running in parallel according to the frequency of natural transfers) and the immune state of the persons through which the strain has passed, and probably on some unknown factors. This may explain why strain

34/57 exhibited a more progressed variation than the other strains at the same time, and why 13/59 was backward.

The IAT, too, shows the variation of the antigenic structure to be progressive. The fact that in the sera absorbed with 12/59, a strain isolated at the end of the period under study, the residual HAI antibody titre was in direct relation to the interval between the isolation of the homologous strain and the isolation of 12/59 suggests that the earlier strains contained from time to time decreasing amounts of the antigenic mosaics lacking in the 12/59 strain. The other part of the experiment illustrated in *Fig. 2* suggests a gradual-development of new antigenic variants. The disappearance of old mosaics seems proceed more rapidly than the appearance of new ones (compare the height of the shaded columns in *Fig. 2/b* to that in *Fig 2/a*).

The continuous progressive variation of the A-2 strains as demonstrated in this paper is very similar to the variation of the A-1 virus. It is therefore likely that the antigenic differences will cause some difficulty in preparing effective vaccines against the A-2 virus.

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Address of the authors:

GYULA TAKÁTSY, KATALIN BARB

State Institute of Hygiene, Gyáli ut 2/6, Budapest IX., Hungary

BACTERIOLOGICAL EXAMINATION OF PATIENTS WITH BRONCHIECTASIS

By

J. SZITA, G. NYIREDY and S. FERENCZY

State Institute of Hygiene, Budapest, Department of Phthisiology, University Medical School,
Budapest, and Central Department for Bronchology of the Municipal Tuberculosis Dispensaries,
Budapest

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Summary. (i) Examination of the bacterial flora in the respiratory tract of patients suffering from bronchiectasis has been performed. The specimens were obtained by use of Kováts's aspirator. The antibiotic sensitivity of the isolated bacteria has been determined.

(ii) From 281 samples from 195 patients 17 different species of bacteria were cultured. Of the 450 strains isolated 39.1 per cent were *Str. viridans* and 15.8 per cent belonged to the *Neisseria* group.

(iii) The bronchial flora of patients with bronchiectasis was not found uniform.

(iv) The best cultural results were obtained by the simultaneous use of direct plating, enrichment in broth and anaerobic cultivation.

(v) With the exception of *Str. viridans* the antibiotic sensitivity of the bacteria isolated corresponded to simultaneous findings in routine material. *Str. viridans* exhibited a resistance higher than usual to streptomycin, penicillin and the broad-spectrum antibiotics. Some strains were resistant even to erythromycin.

(vi) Of the 195 patients a cure was achieved in 86 per cent; improvement was slow in 3 patients; relapses occurred in 17 patients. The condition of 10 patients was unaltered.

(vii) The importance of repeated bacteriological examination and antibiotic therapy based on sensitivity testing *in vitro* is emphasized.

Bronchiectasis is a condition of great importance. In Hungary its incidence among pulmonary diseases ranks second after tuberculosis [12] while in some other countries it already occupies the first place [1].

The role of microorganisms in the pathogenesis of the condition is still unclear, but their responsibility for the aggravation of an existing bronchiectasis is often quite obvious. From the bronchial discharge numerous different organisms have been isolated, such as anaerobic streptococci, staphylococci, pneumococci and *H. influenzae* [9, 15]. Some authors stressed the importance of fusobacterial—spirochaetal infection [5, 8, 17].

Considering the controversies in the literature, with the purpose of obtaining additional data as to the role of microorganisms, we examined bacteriologically the bronchial secretion from patients with verified bronchiectasis, suffering from retention and haemoptysis. The bacteriological studies included determination *in vitro* of the antibiotic sensitivity of the isolated organism.

Materials and methods

Bronchial discharge was obtained by bronchoscopy and a sterile KOVÁTS aspirator [14]. Thus contamination with oral bacteria of the samples was excluded. Bronchoscopy was performed mostly in local anaesthesia, in the morning hours. General anaesthesia was applied in excited patients whose daily sputum did not exceed 50 ml. The sterile bronchoscope tube was introduced, carefully avoiding to touch any organs in the mouth and throat, into the bronchus so that the end of the tube was 2 to 3 cm proximally from the site of sampling. (Eventual secretion in the trachea or the main bronchus was removed prior to bronchoscopy.) The specimen was withdrawn by a sterile aspirator, washed with 5 ml saline into the glass bulb of the apparatus, and distributed into two sterile test tubes for bacteriological and mycological examination. (The mycological findings will be discussed in a subsequent paper.) The sterility of Vofocain solution used for local anaesthesia was regularly controlled. Bacteriological examination of the material was started generally within 5 hours from sampling.

Every material was directly streaked on plates of simple agar, blood agar and chocolate agar and inoculated into broth prepared with Liebig meat extract and into Holman's medium for anaerobic cultivation. The plates were read after an incubation of 18 to 20 hours at 37° C. From the broth enrichment medium the culture was streaked on simple agar, blood agar and chocolate agar plates. Holman's medium was incubated for 3 days, then in case of turbidity or gas production it was opened and the culture was examined by Gram stain. Then the Holman culture was streaked on two blood agar plates; one of the plates was incubated at 37° C for 24 hours aerobically, the other at 37° C for 3 days anaerobically. The sensitivity of the isolated bacteria to 9 different antibiotics was determined, using "Biotest" paper discs (*Institute for Serobacterial Production and Research "Human", Budapest*). The sensitivity of obligatory anaerobes was not tested.

Results

Bacteriology. Out of 281 bronchial samples from 195 patients 13 were sterile. From the remaining 268 samples 450 strains were isolated. Of the samples 132 yielded only one kind of organism. From 95 samples two, from 36 samples three and from 5 samples four different kinds of bacteria were isolated. As in healthy persons and in non-inflammatory diseases of the lung the bronchial system is sterile [7, 14], regardless to whether or not known as normal inhabitants of the throat every isolated organism has been taken into consideration. The results are summarized in *Table I*.

Table I shows that a variety of bacteria may be present in the bronchial secretion of patients with bronchiectasis, as altogether 17 different species were encountered. The incidence of *Str. viridans* and of organisms belonging to the Neisseria group was the highest (39.1 and 15.8 per cent, respectively). As both *Str. viridans* and Neisseriae are the most common inhabitants of the throat, these bacteria have obviously the greatest chance to gain access to the bronchi and to become colonized there. (As already mentioned, there was no possibility of oral contamination of the material.) When *D. pneumoniae* was isolated (5.3 per cent) it almost invariably occurred in pure culture. With some exceptions the strains were of the mucoid form. The incidence of coagulase positive Staphylococci is separately presented (2.9 per cent), while other catalase producing Gram positive cocci are summarized as "other micrococci". The incidence of β -haemolytic Streptococci and *Str. faecalis* was 2.0 and 0.4 per cent, respectively. Other streptococci showing neither α nor β type haemolysis were grouped

Table I

The distribution according to cultural methods of bacteria isolated from the bronchial secretion of patients with bronchiectasis

Cultural methods			B a c t e r i a c u l t u r e d																	
Direct plating	Enrichment in broth	Anaerobic cultivation in Holman medium	<i>Str. viridans</i>	<i>D. pneumoniae</i>	<i>Staph. aureus</i>	Other micrococci	<i>Str. pyogenes</i>	<i>Str. faecalis</i>	Other streptococci	<i>Neisseria</i>	<i>H. influenzae</i>	<i>E. coli</i>	<i>Klebsiella</i>	<i>Proteus</i>	<i>Ps. aeruginosa</i>	Anaerobic cocci	Anaerobic streptococci	<i>F. fusiforme</i>	Non-identified Gram negative rods	Total
+	—	—	1							1										2
—	+	—	18	2	3	6			5	2	1	1	2	1	3				1	45
—	—	+	19	2	2	6	3	1	1			4		1	1	3	1			48
+	+	—	12	5		1	3		4	47	3	1		1				4		77
+	—	+																		
—	+	+	79	5	6	9			19	4		15	5	6	4				2	154
+	+	+	47	10	2	11	3	1	8	17		9	1	3	7				5	124
Totals			176	24	13	33	9	2	37	71	4	30	8	12	15	3	1	4	8	450
Percentage			39.1	5.3	2.9	7.3	2.0	0.4	8.2	15.8	0.9	6.7	1.8	2.7	3.3	0.7	0.2	0.9	1.8	100

as "other streptococci" (8.2 per cent). *H. influenzae* was isolated in 0.9 per cent. Of the Gram negative enteric bacteria *E. coli* occurred in 6.8 per cent, members of the *Proteus* group in 2.7 per cent and *Ps. aeruginosa* in 3.3 per cent. Obligatory anaerobes were seldom isolated (anaerobic cocci 0.7; anaerobic streptococci 0.2; and *F. fusiforme* 0.9 per cent). This finding did not correspond to the results of some other authors who reported on a higher incidence and a larger variety of anaerobic organism. The last column of *Table I* shows the incidence of non-identified Gram negative rods (1.8 per cent).

In evaluating the effectiveness of the various phases of cultivation it should be noted that by direct plating only 203 (2 + 77 + 124) strains were isolated (45.1 per cent). Enrichment in broth yielded further 199 (45 + 154) strains (44.2 per cent). Finally, in the anaerobic medium 48 additional strains were isolated (10.2 per cent). Of the latter, only 7 were obligatory anaerobes. The remaining 41 strains were facultative anaerobic organisms, the anaerobic cultivation of which might be explained on the one hand by the 3 day incubation of the Holman tubes and, on the other, by the adsorption of inhibitory substances (*e. g.* antibiotics) on the cooked meat incorporated in the medium. The adsorptive capacity and improved cultural effect of Holman's medium has been proved by our recent examinations. The low incidence of anaerobic bacteria may have been due to the fact that among the patients only few had pathological changes yielding favourable circumstances for anaerobes (sacculated or capillary bronchiectasis).

Summarizing the results of the various phases of cultivation, by direct plating plus broth enrichment 201 (77 + 124) strains (44.7 per cent), by direct plating plus anaerobic cultivation 124 strains (27.6 per cent), by broth enrichment plus anaerobic cultivation 278 (154 + 124) strains (61.8 per cent) were isolated. Thus broth enrichment together with anaerobic cultivation seemed to yield the best result. The method of choice is, however, to use all the three procedures simultaneously. This ensures optimal bacteriological diagnosis without loss of time.

The isolated strains were tested for antibiotic sensitivity. In *Fig. 1* the antibiotic sensitivity pattern of *Str. viridans* is presented. The other strains have not been included as their number was too small to allow conclusions. It should be noted that the sensitivity of the isolated bacteria was the same as in the routine material of the Department of Bacteriology, State Institute of Hygiene, Budapest.

Fig. 1 shows that the incidence of *Str. viridans* strains resistant to sulphapyridine, penicillin and streptomycin was much higher than one would have expected, and to the broad-spectrum antibiotics they were also markedly resistant. Some *Str. viridans* strains were resistant even to erythromycin. One of the explanations of the high incidence of resistant *Str. viridans* strains might have been that some obstinate cases were examined repeatedly.

Clinical findings. The therapeutical aim in patients with bronchiectasis is to enhance drainage of the secretion and to soothe the inflammation. Drainage of the secretion was promoted by (i) postural drainage (Trendelenburg's position several times daily); (ii) withdrawing the secretion by means of a bronchoscope; (iii) expectorants; (iv) applying proteolytic enzymes (trypsin,

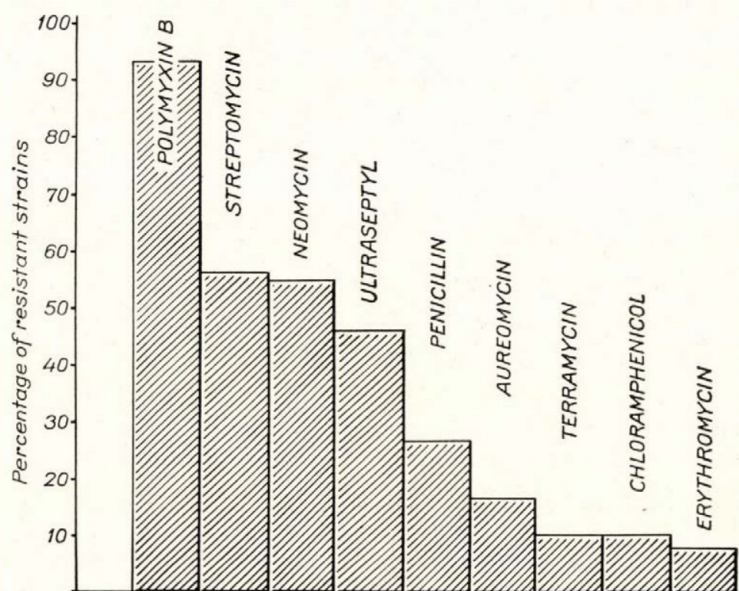


Fig. 1. Percentage frequency according to different antibiotics of resistant *Str. viridans* strains isolated from patients with bronchiectasis

elastase) as aerosols [HUTÁS and NYIREDY, 11] or in cases with thick, adhesive secretion by means of a bronchoscope [KOVÁTS and BAGDY, 13].

In order to eliminate the secretion, antibiotics corresponding to the sensitivity *in vitro* of the implicated organisms were prescribed. An effective treatment frequently used was the application of aerosols containing inhibitory agents. In intractable cases or in bronchorrhoea we had to resort to local treatment by the bronchoscopic method of HORLAY [10] and FERENCZY and NYIREDY [6]. Resistant cases were treated with trypsin aerosol, the bronchoscopic application of cetyl pyridine bromide, suitable antibiotics, and sometimes corticoids.

Of 195 patients 165 became symptomless in 2 to 3 weeks. Recovery was rapid and there was no need to repeat the bronchoscopy. These patients had no relapse in the next 6 to 18 months. In three out of the remaining 30 patients improvement was slow (6 to 8 weeks); they excreted a bacteriologically sterile mucous or serous discharge after treatment had been brought to an end. In spite of aerosol therapy performed at home, 17 patients did not remain symp-

tomless. The condition of 10 patients was unchanged, save for some improvement between relapses. The antibiotic resistance of the bacteria isolated from the latter patients was particularly high. Moreover, during treatment some bacteria which had been sensitive on earlier examinations rapidly acquired resistance to the antibiotics administered, or, in other cases, another kind of resistant flora appeared.

Discussion

As to the bacterial flora in bronchiectasis, most authors believe that pyogenic organisms play an important part in the maintenance and aggravation of the condition [ERMATTINGER, 5; THORPE 18; GREEY, 8]. In contrast to this opinion BUDAY [4], SMITH [17], PILOT *et al.* [16] showed that in bronchiectatic sputum and in the bronchial wall spirochaetae and fusiform bacteria may be present in large numbers. According to PILOT *et al.* [16] these organisms are responsible for the symptoms accompanying bronchiectasis. SMITH, who found spirochaetae and *F. fusiforme* in the sputum of 49 of his 60 patients with bronchiectasis (82 per cent), succeeded in producing artificial bronchiectasis by the respiratory infection of guinea-pigs with material from alveolar pyorrhoea together with the pure cultures of the two organisms mentioned. Other authors stressed the responsibility of *H. influenzae*; BOGGS [3], OPIE *et al.* [15] and BLAKE and CECIL [2] even induced bronchiectasis with *H. influenzae*. From bronchoscopic specimens HARVAKY [9] isolated non-haemolytic streptococci in pure culture.

In the present investigations it has been found that the bacterial flora in bronchiectasis is far from being uniform. As much as 17 different species of organisms were isolated; of these the normal inhabitants of the throat, *Str. viridans* and *Neisseriae*, were the most frequent. This is only natural as they can easily gain access to the dilated bronchi of which the defensive ability is decreased, the physiological function disturbed and where the accumulated secretion provides a good culture medium for bacteria.

The distribution of bacteria in the present material was similar to that observed in chronic bronchitis. This finding, although it does not prove the role of microorganisms in the pathogenesis of bronchiectasis, may suggest that the inflammation and the aggravation of the disease may be attributed to bacterial infection.

The samples were sterile in 13 cases. Out of these in 5 cases the mucous or foamy secretion became clear during therapy. Six of the 13 patients were suffering from dry bronchiectasis. The remaining 2 patients had a sterile, mucopurulent discharge.

Antibiotic therapy based on sensitivity testing *in vitro* was effective in 86 per cent; these patients altogether ceased to produce sputum or their sputum

became clear. The clinical antibiotic resistance generally corresponded to the resistance *in vitro*. The success of antibiotic treatment may be regarded as a proof that most bacteria present in the bronchiectatic discharge are pathogenic agents, so that antibiotic therapy based on bacteriological sensitivity testing may be considered as an important therapeutic factor in bronchiectasis.

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Address of the authors:

JÓZSEF SZITA,

State Institute of Hygiene, Gyáli ut 2/6, Budapest IX., Hungary

GÉZA NYIREDI,

Department of Phthysiology, University Medical School, Diósárok ut 1, Budapest XII., Hungary

SÁNDOR FERENCZY,

Central Department for Bronchology of the Municipal Tuberculosis Dispensaries, Diósárok ut 1, Budapest XII., Hungary

FURTHER STUDIES ON THE CYTOPATHOGENIC EFFECT OF ADENOVIRUSES IN HUMAN AMNIOTIC CELL CULTURES

By

I. NÁSZ and MARGARET TÓTH

Institute of Microbiology, University Medical School, Budapest

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Summary. The characteristics of the cytopathogenic effects of the first 7 types of adenovirus were studied on human amniotic cell cultures. The results of our earlier studies were further supported by demonstrating some additional characteristic features in the cytopathogenic effects, particularly in those caused by type 3 and 5 adenoviruses. The other strains had either an intermediary type of cytopathogenic effect, or one similar to that of type 5. As cytopathogenic changes were usually present in both the cytoplasm and the nuclei, the differences observed are described for these two parts of the cells separately.

In an earlier paper we reported about some characteristics of the cytopathogenic effects exerted by the first 7 types of adenovirus in human amniotic cell cultures [1]. The virus strains studied were divided into two groups according to the type of cytopathogenic effect produced. Type 3 and type 5 strains were regarded as prototypes. These two main types of the cytopathologic change were termed "type 3 degeneration" and "type 5 degeneration".

To obtain further information about the two types of degeneration it seemed desirable to study not only the differences in the general appearance of the infected cultures but also those present in the cells themselves.

Materials and methods

Virus strains. The same strains were used in these studies as in the previous ones [1]. The strains were maintained and titrated on Detroit-6 tissue cultures. For morphological studies the amniotic cells were infected with 10^4 TCID₅₀ of the appropriate virus strain.

Tissue cultures. The methods used for preparing, maintaining and infecting both Detroit-6 and amniotic cell cultures have been described [1, 2]. The nutrient medium used consisted of Hanks' solution containing 0.5 per cent lactalbumin hydrolysate and 20 per cent human serum. For maintenance, Hanks' solution containing 0.5 per cent lactalbumin hydrolysate and 5 per cent rabbit serum was used. Appropriate amounts of penicillin, streptomycin and mycostatin were added to each medium.

For morphological examination cells cultivated on floating coverglasses were used. The coverglasses were placed into the tubes simultaneously with the addition of the cell suspension.

Sampling. This was made essentially according to the method described earlier for human embryonic cell cultures [3, 4]. Samples of the coverglass cultures were taken every 10 to 14 hours during a period of 7 to 10 days. The samples were fixed in methyl alcohol and stained with Giemsa's solution. In some cases the Feulgen reaction was also carried out.

Control cultures were also examined. Their medium was changed either to maintenance solution or to killed virus suspension at the time of infecting the experimental cultures.

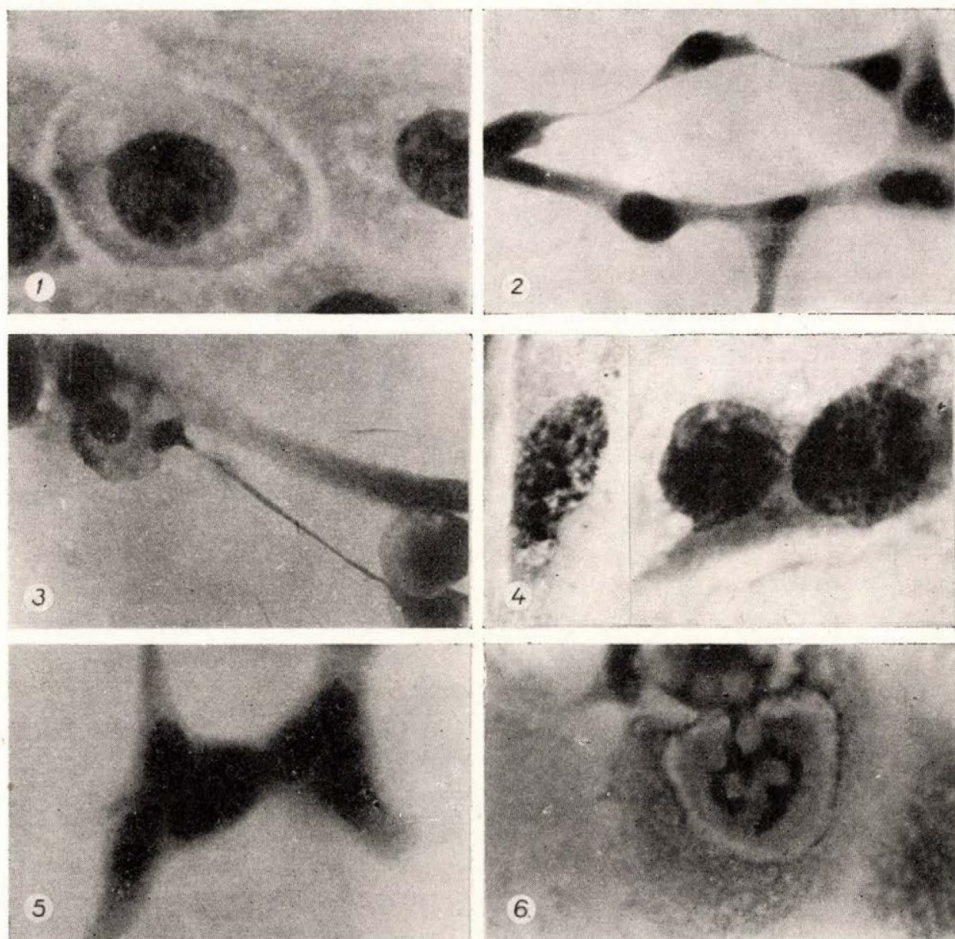
Results

The pathomorphological characterization of the cytopathologic events induced by the first 7 types of adenovirus will be discussed in their relation to type 3 and type 5 degeneration, these being the most different from each other. As pathological changes were present not only in the nuclei [5, 8] but also in the protoplasm, the differences between the two types of degeneration were studied in both of these cell components.

Type 3 degeneration. This was characterized by an initial rounding up and shrinking of cells 1 to 2 days after the infection. This procedure extended later from the periphery towards the central area of the cell layer resulting in a network-like transformation of the culture [8]. At the same time the cells involved were rounded up so that their protoplasm appeared as a thin, sharp-bordered halo around the nucleus. The cells were connected by elongated protoplasmic threads resulting in the development of the network-like pattern consisting of cells arranged in chain or ring-like formations (*Fig. 1* and *2*). Later this pattern was disrupted but the protoplasmic threads persisted (*Fig. 2* and *3*). These network element appeared to consist of two different types of structural units, 1. chains of cells with a protoplasmic ring hardly more in diameter than the nucleus; 2. degenerated cells connected with one another by elongated thin protoplasmic bridges.

Simultaneously with the changes in the general appearance of the cultures granulation appeared in the nuclei (*Fig. 4*) which soon became general. Though in some exceptional cases a central mass or some other morphological units might have been present in the nuclei, it was the diffuse granulation that appeared to be the most general feature. Parallel to the breaking down of the cellular-network and the formation of small groups of degenerated cells, the cell borders successively disappeared and the amount of nuclear material gained remarkable preponderance over the protoplasmic residues (*Fig. 5*). For a short period nuclear and protoplasmic material could still be differentiated by Giemsa's stain while with the progress of shrinking nothing but a homogeneous basophilic mass was left over from the cells.

Type 5 degeneration. This was characterized by a heavy granulation of the cells and disappearance of the cell borders, resulting in the formation of single-standing degenerated cells [8]. The details of this procedure were as follows. 24 to 48 hours after the infection began the granulation of nuclei. The small granules aggregated into different shapes, such as crescents (*Fig. 6*), rings (*Fig. 7*) or central masses (*Fig. 8*) inside the nuclear membrane. In addition to these most frequently occurring shapes a wide variety of different formations may occur. In the early phases of degeneration the nucleoli could still be differentiated, but later they vanished. The nuclei involved exhibited around the above-described intranuclear bodies a glassy bright



Explanations to Figures. All the photographs present primary human amniotic cell cultures. All preparations but that in Fig. 8 were stained with Giemsa's solution; Fig. 8 represents a Feulgen reaction

Fig. 1. Uninfected normal cell 4 days after the addition of maintenance solution. Magnification about $\times 1600$

Fig. 2. Cells 96 hours after infection with type 3 adenovirus. Magnification about $\times 650$

Fig. 3. Cells 104 hours after infection with type 3 adenovirus. Magnification about $\times 700$

Fig. 4. Cells 110 hours after infection with type 3 adenovirus. Diffusely granulated nuclei. Magnification about $\times 1350$

Fig. 5. Cells 120 hours after infection with type 3 adenovirus. Degenerated cells arranged in groups. Magnification about $\times 750$

Fig. 6. Cells 53 hours after infection with type 5 adenovirus. Semicircle-like central structure in the nucleus. Magnification about $\times 1350$

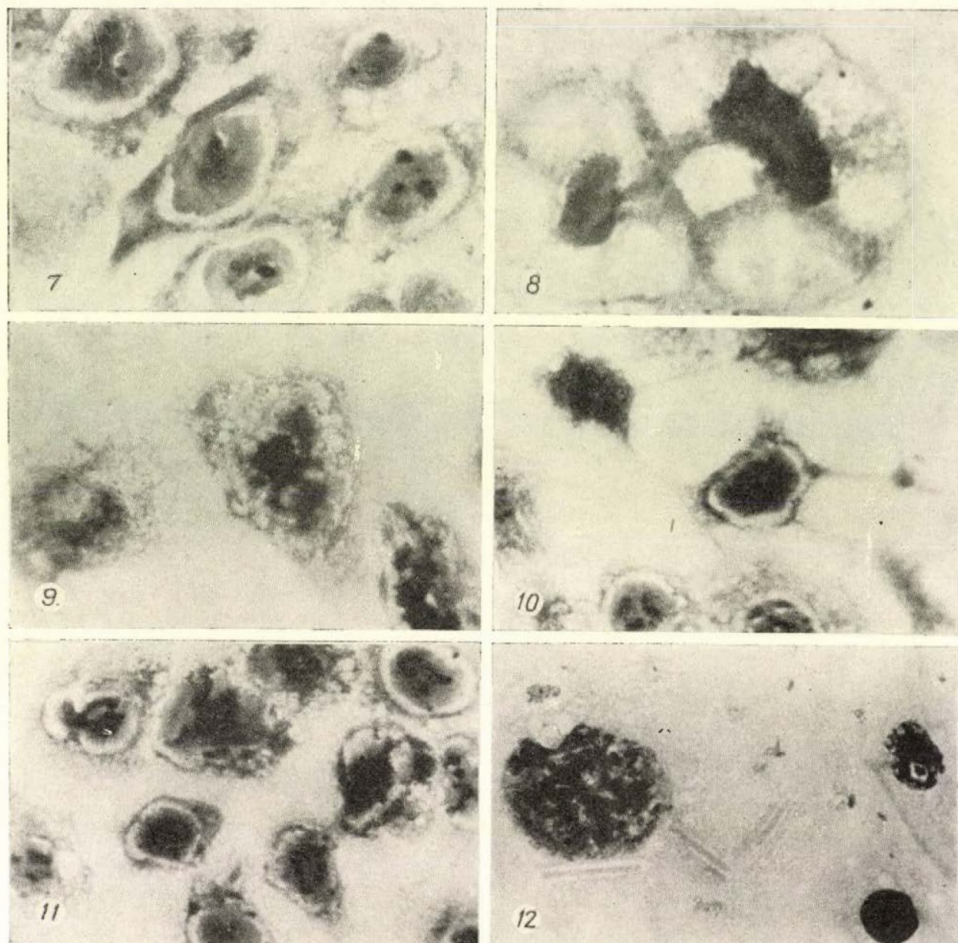


Fig. 7. Cells 53 hours after infection with type 5 adenovirus. The different central structure and the glassy background are clearly seen in the nuclei surrounded by loose peripheral material. Magnification about $\times 1300$

Fig. 8. Cells 134 hours after infection with type 5 adenovirus, with a Feulgen positive central mass in the nuclei. Magnification about $\times 2100$

Fig. 9. Cells infected with type 5 adenovirus exhibiting central mass and dissolved nuclear membrane. Magnification about $\times 1300$

Fig. 10. Cells 72 hours after infection with type 5 adenovirus. Magnification about $\times 850$

Fig. 11. Cells 80 hours after infection with type 5 adenovirus. Magnification about $\times 850$

Fig. 12. Cells 110 hours after infection with type 7 adenovirus. Crystalline formations in the cytoplasm. Magnification about $\times 1350$

mass, often separated from the nuclear membrane by a thin, non-staining peripheral ring (*Fig. 7*). Simultaneously with the formation of the central mass in the nucleus, the border between nucleus and protoplasm sometimes became indistinct (*Fig. 9*), though as a rule the nuclear membrane remained discernible for a long periods. Swelling and deformation of the nuclei involved was frequent.

Parallel to the changes in the nucleus degenerative procedures occurred in the protoplasm. After the destruction of the cell layer the cytoplasm of the single cells became loose with indistinct borders, exhibiting a granulated and foamy structure breaking successively off from the nucleus. Numerous cells with the above-described typical nuclear damage exhibited only a thin layer of cytoplasm, which sometimes was still in connection with the neighbouring cells (*Fig. 10*). Later on the whole culture was transformed to separate degenerated nuclei surrounded by protoplasmic residues with blurred outlines (*Fig. 11*). At the final stage these cells, too, turned into homogeneously basophilic bodies with no internal structure.

Both in the protoplasm and the nuclei of infected cells there were often some crystalline structures of undefined origin (*Fig. 12*).

For all the other 5 strains examined the morphology of the damaged cells was mostly similar to the type 5 degeneration or in some to type 3 degeneration, with a variety of different intermediary forms. No similar pathologic changes arose if the cells had been treated with a killed virus suspension.

Preparations stained with Giemsa's solution disclosed how in most instances the initial eosinophilia was changing into basophilia, parallel to the proceeding of the degeneration. The morphological elements in the damaged nuclei were usually Feulgen positive (*Fig. 8*).

Discussion

Prior to the present studies several authors had examined the characteristics of the cytopathologic changes caused by the different strains of adenoviruses [5—14]. These studies revealed remarkable differences in cytopathogenic effect of the different strains [5, 7, 8, 10]. Some authors [5, 8] made attempts to group the sequence of the pathological events in certain chronological phases. The data presented above make the value of such a classification questionable, considering the simultaneous presence in the preparations of a wide variety of degenerative changes at different points of time. This suggests that the degenerative procedures did not develop parallel in all the cells of the same culture. The question naturally arises whether the simultaneous cellular changes represented different phases of the same infection, or there was a different morphological response to the same infection in certain cells. We are inclined to regard

the former to be more probable, though there are no decisive data to exclude the second possibility.

Also, as to the nature of the different morphological units formed in the nuclei of infected cells, we can rely only on suppositions. Nevertheless, the lack of similar changes after treatment with killed virus, the change of eosinophilia into basophilia, and the positive Feulgen reaction suggest an increased desoxyribose nucleic acid content of the nuclei, probably owing to virus reproduction [5]. Thus at least, part of the different structures present in the damaged nuclei might be in connection with virus synthesis.

As to the nature of the crystal-like structures observed in different parts of infected cells, we agree with LEUCHTENBERGER *et al.* [9], who examined the crystals present in HeLa cells infected with type 3 and 5 adenoviruses. The presence of the crystals in living infected cells and their absence in both native and fixed stained control preparations permit to exclude the supposition that they might be artefacts. Thus it seems to be highly probable that these crystals play an important role in the infectious process though it is not clear whether they represent the virus itself or a special phase of virus production, or probably a product of a host-cell interaction.

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Address of the authors:

ISTVÁN NÁSZ, MARGIT TÓTH

Institute of Microbiology, University Medical School, Hőgyes E. u. 9, Budapest XII., Hungary

AN EPIDEMIC OF BORNHOLM DISEASE IN HUNGARY IN 1958

By

I. DÖMÖK, ELIZABETH MOLNÁR and O. RUDNAI

State Institute of Hygiene, Budapest

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Summary. A Bornholm epidemic raging in Hungary during the summer and autumn of 1958, which spread from west to east and involved more than 40 000 persons, with the highest incidence among children under 15 years of age, and causing myocarditis and meningoencephalitis among newborns in two maternity wards, are described.

Employing the monkey-kidney tissue culture and/or suckling-mouse technique, virus isolations were attempted in 965 specimens from 720 patients. Altogether 308 virus strains were isolated from 304 samples taken from 273 persons; 265 of the strains were Cocksackie B3.

The aetiological role of this viral type in the epidemic has been confirmed by neutralization tests with sera from 300 patients; many of these were paired sera.

Cocksackie B3 virus appears to have been responsible for several cases of aseptic meningitis and febrile episodes, and probably, to be capable of causing herpangina.

The Bornholm disease manifested itself mostly with abdominal symptoms among children and thoracic symptoms among adults. The white cell count was normal in 78 per cent but the erythrocyte sedimentation rate in only 40 per cent of the subjects tested.

Most of the patients excreted the virus for a long time after recovery, but newborns generally for not more than two weeks.

Some of the Cocksackie B3 strains could only be isolated in monkey kidney tissue cultures, others in suckling mice. The latter variants occurred more frequently in material from newborns.

In the year of the Bornholm epidemic reviewed in this paper, the incidence of poliomyelitis was the lowest observed in Hungary over a 20-year period. This was probably due to an antagonism between Cocksackie B and poliomyelitis viruses.

For some years research work on an imposing scale has been in progress in all parts of the world to establish the aetiological role of enteroviruses. The results point, among other things, to essential differences between Cocksackie (C) Group A and Group B viruses, not only in the histological lesions they produce in suckling mice but also in the part they play in human pathology. From the latter point of view great significance attaches to C B viruses, for these are considered the cause of Bornholm disease [1] and of the grave syndrome generally referred to as neonatal myocarditis [2].

The observations reported in the present paper afford additional evidence linking C B viruses with these two diseases.

In the summer of 1958, an epidemic of Bornholm disease broke out in Hungary, vaster than any ever yet reported in the literature. While it was spreading practically all over the country, a number of newborns were found to have fallen seriously ill at several obstetrical departments. A particularly vehement outbreak in one of them has been reported in detail elsewhere [3-5].

The following is an account of the epidemiological observations made throughout the outbreak and of results obtained in virus isolation and antibody studies on specimens collected during and after the epidemic.

Epidemiology

Case-locating methods. In Hungary Bornholm disease is not reportable wherefore the following measures were taken by this Institute to secure as much information as possible about the epidemic. (i) An appeal [6] was published requesting all practitioners to supply information on every occurrence in their respective area. (ii) The country-wide network of the Public Health and Epidemiological Stations was alerted. All stations were instructed to prepare special reports from the notes of the district physicians and dispensaries on the occurrence of and course run by the disease, and to submit them regularly to this Institute. (iii) Special survey squads were sent into several affected areas to obtain reliable details of incidence and characteristic symptoms.

Outbreak, spread and duration. Sporadic cases were diagnosed in some areas (*e. g.* in County II*) as early as February, 1958. The epidemic proper began in May in County I, in the southwestern part of Hungary, whence it spread to the middle part, and from there to the eastern region. In the western areas it reached its peak on the whole in July, and in the eastern areas in August. After that it gradually receded, and was over by the end of September except for sporadic cases reported later mainly from the eastern parts of the country. The number of cases was much higher in the western than in the eastern region. In the latter, unlike in the former, only focal outbreaks occurred.

Epidemiography by districts. In Transdanubia — the part of the country west of the Danube — the epidemic was most intense in Counties I and II, where it had originally started. In County I, 6659 cases of pleurodynia and abdominal pain, respectively, were diagnosed by district physicians and in the specialist dispensaries from June 1 to September 20. In one district, having a population of 6500, as many as 400 patients presented themselves with symptoms characteristic of Bornholm disease in the period from the beginning of May to July 23. Outbreaks of similar proportions were reported from six additional districts of this country, where the incidence rate for most localities generally varied between 5 and 6 per cent, though in some communities in the valleys of the Zala and Mura rivers even 50 to 60 per cent of the inhabitants were afflicted.

In County II the disease was first observed to have attained heavy proportions at the beginning of June, and mostly in the west, *i. e.* the districts bordering on County I. Somewhat later cases were most numerous in the northern parts bordering on Lake Balaton. In July and August about 14 000 cases were diagnosed in this county as Bornholm disease, and in August alone as many as 1000 people were allowed sickness benefit.

* See Fig. 1.

Symbols : ■ excretors of Coxsackie B3 virus
 □ excretors of other enteric viruses
 ▣ excretors of Coxsackie B3 and other enteroviruses

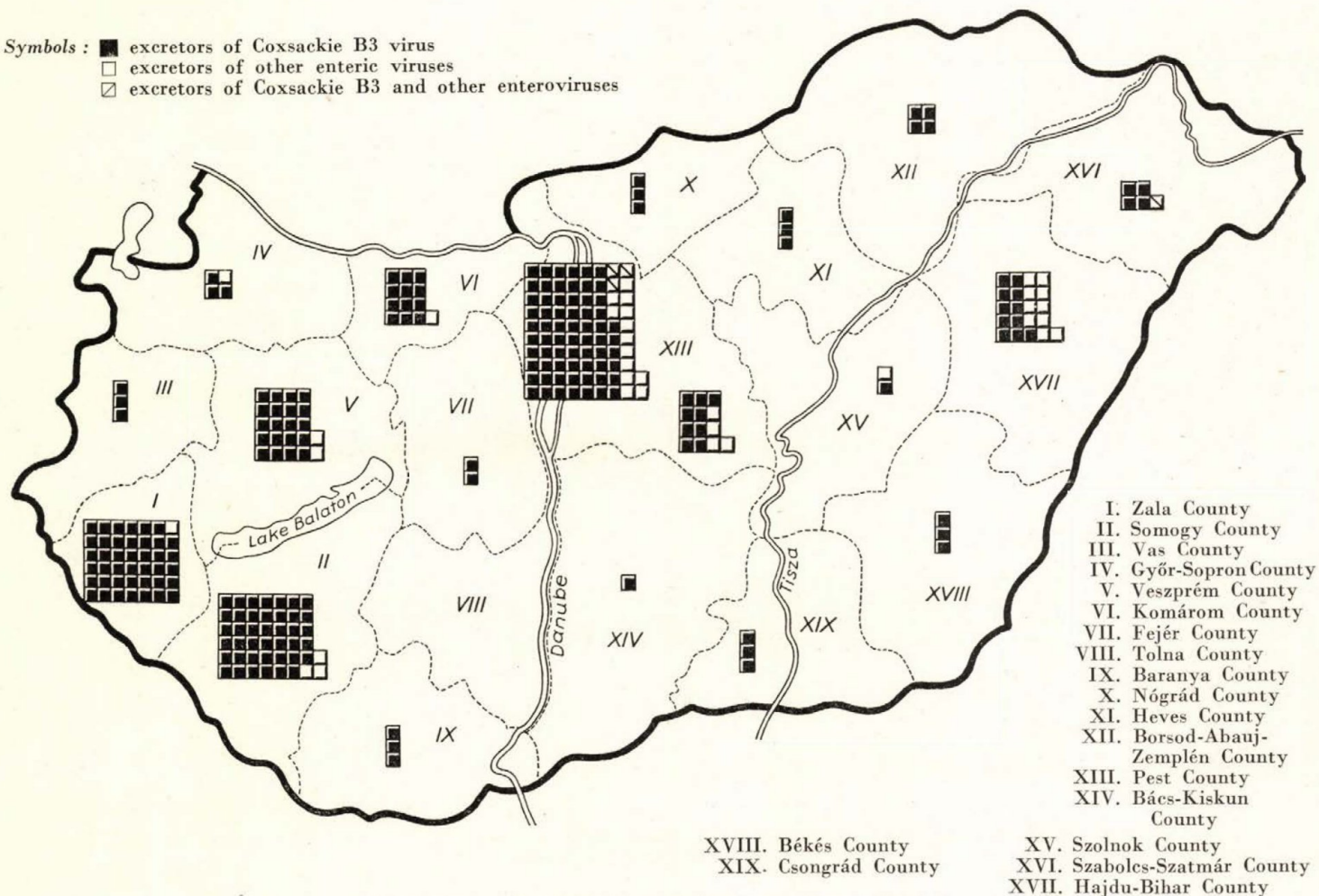


Fig. 1. Geographical distribution of virus-positive individuals

In the other counties of Transdanubia (III—IX) the disease developed into an epidemic largely in the second half of June, with from 3000 to 5000 cases diagnosed in each. From initial minor foci in and around individual villages the disease gradually spread to the larger towns. Thus, for instance, in Szombathely (County III) by the end of July from 15 to 20 per cent of the patients on the sick-lists of the district physicians and dispensaries in the town's three health districts were suffering from Bornholm disease. For the two months of August and September this proportion was still 14.2 per cent (846 out of 5952). In Veszprém (County IV) the cases reported numbered 750. In the town of Tatabánya (County VI) 1200 Bornholm disease diagnoses were registered from mid-June to mid-August.

In the central part of the country the epidemic appeared as late as August. Critical areas were Counties X and XII in the north, with 4000 and 3500 cases, respectively. It merits mentioning that in County X localities along the railway lines were chiefly involved. In Budapest and in the Counties XIII and XIV there were only sporadic cases.

In the eastern region of Hungary — the Counties beyond the Tisza River (XV—XIX) — there were mostly sporadic cases. A few epidemic foci, however, developed also in these parts. In Csabacsüd — a village of 3000 inhabitants in County XVIII — 80 cases were diagnosed in a single month. In one district of County XIX and in the towns Szentes and Csongrád 500 persons developed symptoms characteristic of Bornholm disease.

Total number of cases. The methods described above yielded more than 40 000 diagnosed cases. But as these measures had not been sufficiently effectual to produce information of each and every case observed by the physicians and as many milder cases were probably never seen by a physician, the total number of cases for the whole country was probably much higher.

Distribution within families and contacts. In this respect our observations agreed with those of a number of earlier authors [e. g. 7—10]. In County II there were some 400 families in which two, and 80 families in which more than two members had been taken ill. In some close groups all or nearly all contacts contracted the infection. In County I, for instance, in a Kindergarten for 36, all the children became affected. In a holiday home on Lake Balaton the epidemic involved almost all the children in the 2 to 14-year age group and most of the adults.

Distribution by age. About half of the cases observed occurred in children under 15 years of age, which represents 26 per cent of the country's total population. This clearly points to a higher frequency among children than among adults. It is noteworthy, however, that with minor symptoms less adults than children sought medical assistance.

Occurrence among newborns. While the epidemic was raging in the country there was an accumulation of cases among newborns in two obstetrical insti-

tutions. In a number of other similar institutions sporadic cases were only observed. One of the two major hospital outbreaks occurred in the maternity ward of the General Hospital in Tatabánya (County VI), the other in the 2nd Obstetric Department of the University Medical School, Budapest. In the first, nine cases were diagnosed, all between August 17 and 31, of which one ended fatally. All displayed clinical symptoms indicative of myocarditis. To this maternity ward, a few days before the outbreak a pregnant woman suspected of premature delivery had been admitted whose pains, first thought to be pains of labour, subsequently turned out to be of pleurodynic origin. This patient probably was the original source of infection. In the 2nd Obstetric Department in Budapest 22 cases were diagnosed between September 9 and 26, revealing chiefly nervous symptoms. Three newborns succumbed to the disease. Three mothers displaying no symptoms tested after their babies had fallen ill were found to excrete in their faeces the agent responsible for the Bornholm epidemic. Obviously, in this place more than one infective source had been at work [4].

Aetiology

Materials and methods

Specimens for examination. Usually these were sent by mail to this laboratory. For virus isolation mostly faecal samples were received. From patients with central nervous system lesions, and sometimes also from subjects afflicted with some other illness, CSF was obtained. In a number of cases throat swabs and section material were put at our disposal, chiefly from newborns. In addition, acute and/or convalescent blood samples were submitted from many patients for the determination of the antibody response.

Preparation of specimens. This was done with the method described in an earlier paper [4]. Nystatin was used to avoid mycotic contamination. Blood samples were centrifuged on arrival; the sera were drawn into rubber-stoppered vials, inactivated at 56° C for 30 minutes, and stored at -30° C just like material prepared for isolations.

Isolation tests were carried out either in monkey kidney tissue cultures or in suckling mice. Many specimens that turned out to be negative with one technique were then tested with the other.

a) *Monkey-kidney tissue cultures* were prepared according to YOUNGNER [11]. Three cultures each were inoculated with 0.1 ml of the same specimen and incubated at 37° C for 20 to 30 minutes, whereafter 0.9 ml of fresh nutrient fluid were measured into each tube. The infected tissue cultures were then incubated at 37° C for 14 days, but on the seventh day the fluid in all negative cultures was replaced by fresh medium consisting of equal volumes of Hanks' solution and Parker's 199 medium. The cultures were examined daily.

b) *The suckling mice* used were one-day-old animals of our Institute's breed. A quantity of 0.03 ml of the same specimen was inoculated subcutaneously into each of a group of eight mice, which were then observed for ten days. Passages in mice were performed as described in an earlier paper [12].

Identification. Apart from a few exceptions, viral agents were identified by neutralization test in monkey-kidney tissue culture, even the strains isolated in suckling mice, provided symptoms characteristic of C B viruses had developed in the mice.

The first ten strains isolated were simultaneously tested with specific antisera prepared in mice against each of the five types of C B virus. Since ultimately all of them proved to be of the B3 type, all strains isolated in the later phase of the epidemic were first typed against this particular type serum. If this failed to neutralize identification was attempted against the following type sera or combinations of them: poliovirus type 1, 2 and 3; ECHO type 1 to 10, 12 and 14; C A9 and B1 to 5.

Strains giving rise in the mice to signs characteristic of C A virus infection were neutralized against A 1 to 13 type sera in suckling mice.

In neutralization tests a fixed amount of a suspension of the isolate diluted 1:50 or 1:500 was mixed with an equal amount of the appropriately diluted type sera described below and normal mouse serum, respectively. Following incubation at 37° C for one hour, 0.1 ml amounts of these mixtures were separately inoculated into each of three tissue cultures or 0.03 ml quantities into each of eight suckling mice. Tissue cultures and groups of animals showing neutralization, were kept under observation for at least an additional four days following the full development of the lesions seen in the controls.

Standard virus strains. The A1 to 10 and B1 to 4 type strains were kindly supplied by G. DALLDORF, New York. For the Coxsackie A11 to 13 and B5 types as well as the ECHO type strains we are indebted to M. P. CHUMAKOV, Moscow.

Type sera. The poliomyelitis 1 to 3 type sera were received from H. B. MAITLAND, Manchester.

C type sera were obtained by immunizing mice of 12 to 14 g weight with type strains in the following manner. Of a 20 per cent infected mouse torso suspension 0.25 ml amounts were intraperitoneally injected into a number of mice on three occasions two or three days apart, and a fourth injection was given one month following the third. On the 7th to 10th day after the last injection the animals were bled to death. Inactivated sera were used in a final dilution of 1:10.

ECHO type sera were prepared in rabbits weighing 2.5 to 3 kg. These were inoculated intravenously with 4 to 5 ml of a suspension of type strains derived from monkey-kidney tissue culture, on four occasions at the same intervals as the mice. In neutralization tests the inactivated sera were used in 1:25 or 1:50 dilutions. All type sera were distributed into vials and stored at -30° C.

C B3 neutralizing antibodies were established in human sera with the colour test [13] against 100 TCD₅₀ of the virus. In preliminary tests the sera were first examined at a 1:5 dilution for their respective antibody content. Those that proved positive at this dilution were subjected to further tests employing 3 1/2-fold serial dilutions. The end dilution in the first tube of the series was 1:7. TAKÁTSY's 0.1 ml spiral loop [14] was used in preparing the serum dilutions. Each serum dilution was examined in two parallel tubes. The cells of a passable monkey-heart culture, kindly supplied by M. P. CHUMAKOV, Moscow, were used in the test. The test was performed in rubber-stoppered Widal tubes. To 0.25 ml of serum dilution 0.25 ml of virus dilution (containing 200 TCD₅₀) were measured; this was overlaid with 0.6 ml of paraffin oil and incubated for one hour at 37° C whereafter 0.25 ml of cell suspension, containing 120 000 cells per ml, were added to it. The fluid to dilute this mixture of serum, virus, and cell suspension was composed as follows: Parker's Medium No 199, 41.5%; Hanks's solution 41.5%; 5% lactalbumin hydrolyzate, 10%; 20% glucose, 2%; Calf serum 5%, with 100 I. U. of penicillin, 0.2 mg of streptomycin and 100 I. U. of Nystatin added per each ml. The pH was adjusted to 7.5 with 5.6% NaHCO₃.

Final readings were taken between the 7th and 10th day. As end-titre that serum dilution was regarded at which the pH in one of the two parallel tubes showed a shift towards acidity. Every test was subjected to three different controls, viz. virus titration, control of 100 TCD₅₀ of virus, and tissue control.

Essential information concerning each case was collected on special forms handled routinely in the Diagnostics Laboratory of this Institute's Virus Department. In addition to the patient's personal data these forms recorded the clinical diagnosis, the characteristic symptoms, all clinical laboratory findings, the date of onset, and the dates of samples taken.

Results

Number of individuals tested for enteroviruses and of isolations obtained from them during the summer and autumn. In addition to substantiating the aetiology of the cases diagnosed in the reports as typical of Borholm disease, the examinations carried out by us during the epidemic (June 1 to September 30, 1958) and postepidemic period (October 1 to December 31, 1958) aimed at ascertaining the part played by the causative agent of the epidemic in the

occurrence of other diseases and at establishing the frequency rate of the other enteroviruses. The cases were distributed into seven groups according to the clinical symptoms reported and the isolations were set against the respective syndrome groups (Tables I and II). Group I comprised all patients reported as suffering from typical Bornholm disease, Group II the newborns and premature infants who displayed signs of meningoencephalitis, myocarditis or common cold. Most of them, together with their apparently normal mothers and baby ward-mates classed with Group III, have been included in these examinations in connection with the two hospital outbreaks mentioned. From one of these outbreaks, material from 9 affected and 3 symptom-free newborns, and from the other, specimens from 17 affected, 2 symptom-free newborns and 15 mothers, were examined [4]. In Group IV were classed subjects in whom clinically nothing more appeared than angina and fever. Group V embraces the cases with the diagnosis of herpangina. The conditions combined in Group VI under the heading "CNS lesions" were mostly cases of aseptic meningitis, with a few of meningoencephalitis, encephalitis, paralytic poliomyelitis, and polyradiculitis. In Group VII were placed all the cases (*e. g.*, enteritis, atypical exanthema, etc.) which could not be classed with either of the preceding groups.

It can be seen from *Table Ia* that during the epidemic 511 persons were examined virologically, of whom 242 turned out to be positive: 218 for C B3 virus, 20 for other enteroviruses, and 4 were found to have attracted dual infection. C B3 virus was isolated from 67.7 per cent of the 242 persons tentatively diagnosed in the reports suffering from Bornholm disease (Group I); but other viruses were recovered from 2.5 per cent only. Of the 32 newborns in Group II a similarly high proportion was C B3 positive: 21 patients, or 66 per cent. While isolations were relatively rare in the remaining five groups, it should not be overlooked that in Groups IV to VII the total number of C B3 positives (37) greatly exceeded that of all the other virus isolations (17). If account is taken of the fact that 6 of the 20 symptomless individuals in contact with affected newborns (Group III) were found to be infected with C B3, it seems legitimate to assume that latent infections were frequent throughout the epidemic period.

Table Ib shows that in the postepidemic period 209 persons were examined virologically, of whom only 31 patients, or 15 per cent, were positive. Of these 31 virus-positive cases 15 still harboured C B3 strains, that is, almost as many as were carriers of all the other enteroviruses. Yet, the percentage rate of C B3 isolations from all persons tested decreased from 43.4 during the epidemic to 7.1 in the postepidemic period, while the isolation rate of all the other enteroviruses rose from 4.7 to 7.6 per cent. In none of our groups was the isolation rate high not even in the group of typical Bornholm disease; obviously, many more cases had been erroneously diagnosed by the physicians

Table I

Number of individuals tested for enteroviruses and of isolations obtained from them

a) during the epidemic period (from June 1 to September 30, 1958)

Disease group	Number of					
	indi- viduals tested	isolations of				%
		C B3 virus	other entero- viruses	C B 3 plus other ent. vir.	Total	
I. Bornholm disease	242	164	6	—	170	70
II. Meningoencephalomyocarditis neonatorum ..	32	20	—	1	21	66
III. Environmental contacts with newborns ...	20	6	—	—	6	30
IV. Febrile episodes	43	7	3	—	10	23
V. Herpangina	15	3	—	1	4	27
VI. Lesions of central nervous system	115	16	10	2	28	24
VII. Miscellaneous conditions	44	2	1	—	3	7
Total	511	218	20	4	242	47

b) after the epidemic (from October 1 to December 31, 1958)

I. Bornholm disease	19	5	—	—	5	26
II. Meningoencephalomyocarditis neonatorum ..	2	1	—	—	1	—
IV. Febrile episodes	36	—	6	—	6	17
V. Herpangina	4	—	—	—	—	—
VI. Lesions of central nervous system	92	9	8	—	17	18
VII. Miscellaneous conditions	56	—	2	—	2	4
Total	209	15	16	—	31	15

in the postepidemic period than during the epidemic period. While during the epidemic three quarters of the strains isolated from patients classed with Group IV for „febrile episodes” were C B3 strains, not one of those isolated after the epidemic was of this type. In the latter period C B3 was, however, isolated from a number of cases listed as “CNS lesions” in Group VI.

Number of specimens tested for enteroviruses and of isolations obtained from them during and after the epidemic. During the epidemic 661 specimens originating from 511 individuals were tested for the presence of enteroviruses (Table II/a). Of these, 269 samples yielded 273 strains, of which 91 per cent were of the C B3 type and only 9 per cent belonged to another B type, or to the C A group, or to some other virus. The overwhelming majority of the specimens having been faeces, it was from these that viral strains were obtained in the highest number. Of the 112 samples of CSF only 8 contained some kind of a virus strain. Of the 26 other specimens all the positives yielded C B3 virus; 4

Table II

Number of specimens tested for enteroviruses and of isolations obtained from them

a) during the epidemic (from June 1 to September 30, 1958)

Disease group	Specimens tested			Type of virus isolated																
	type	num- ber	found posi- tive	Coxsackie						ECHO					Polio			Mixed strains	Uniden- ti- fied	
				A5	A6	B1	B2	B3	B4	1	7	8	9	14	1	2	3			
I. Bornholm disease	Stools	241	167	—	—	1	1	161	1	—	—	1	1	—	—	—	1	—	—	
	CSF	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	Miscell.	4	4	—	—	—	—	4	—	—	—	—	—	—	—	—	—	—	—	
II. Meningoencephalomyo- carditis neonatorum	Stools	50	26	—	—	—	—	26	—	—	—	—	—	—	—	—	—	—	—	
	CSF	18	4	—	—	—	—	3	—	—	—	—	—	—	—	—	—	E1*+CB3	—	
	Miscell.	20	16	—	—	—	—	16	—	—	—	—	—	—	—	—	—	—	—	
III. Environmental contacts with newborns	Stools	20	6	—	—	—	—	6	—	—	—	—	—	—	—	—	—	—	—	
IV. Febrile episodes	Stools	42	10	—	1	—	—	7	—	—	—	—	1	—	—	—	—	—	1	
	CSF	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
V. Herpangina	Stools	15	4	—	—	—	—	3	—	—	—	—	—	—	—	—	—	CA5+CB3	—	
VI. Lesions of central nerv- ous system	Stools	111	25	1	—	—	1	14	—	—	1	—	1	1	2	1	—	P1**+CB3, CA2+CB3	1	
	CSF	84	4	—	—	—	—	3	—	—	—	—	—	—	—	—	—	—	1	
VII. Miscellaneous conditions	Stools	44	3	—	—	—	—	2	—	1	—	—	—	—	—	—	—	—	—	
	CSF	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	Miscell.	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Total	Stools	523	241	1	1	1	2	219	1	1	1	1	3	1	2	1	1	3×2 strains	2	
	CSF	112	8	—	—	—	—	6	—	—	—	—	—	—	—	—	—	1×2 strains	1	
	Miscell.	26	20	—	—	—	—	20	—	—	—	—	—	—	—	—	—	—	—	

b) after the epidemic (from October 1 to December 31, 1958)

I. Bornholm disease	Stools	22	5	—	—	—	—	5	—	—	—	—	—	—	—	—	—	—	—
II. Meningoencephalomyo- carditis neonatorum . . .	Stools	2	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—
IV. Febrile episodes	Stools	36	6	—	—	—	5	—	—	—	—	—	—	—	—	—	—	—	1
	CSF	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Miscell.	11	1	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—
V. Herpangina	Stools	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Miscell.	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
VI. Lesions of central nerv- ous system	Stools	91	18	—	—	—	—	9	—	—	—	1	2	—	2	—	—	—	4
	CSF	67	2	—	—	—	—	1	—	—	—	1	—	—	—	—	—	—	—
VII. Miscellaneous conditions	Stools	53	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2
	CSF	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Miscell.	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total	Stools	208	32	—	—	—	5	15	—	—	—	1	2	—	2	—	—	—	7
	CSF	76	2	—	—	—	—	1	—	—	—	1	—	—	—	—	—	—	—
	Miscell.	20	1	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—

* ECHO 1 strain

** Poliomyelitis Type 1 strain

were throat swabs from Bornholm patients and all were positive; 6 were tracheal secretions from ill premature infants and 2 of them were positive; 14 specimens — sections from the CNS, heart, lung, pancreas, liver, spleen and intestines — from 2 newborns who had died a sudden death, were all positive at high titres; 2 post mortem specimens of liver and spleen from adults proved negative.

With the exception of one, all the specimens from newborns yielded C B3 strains only. The isolations obtained from CSF and CNS samples point to the participation of the CNS; in one of the newborns its participation has been proved histologically [3, 5]. While the faeces of one newborn harboured a C B3 strain only, its CSF yielded both this and an ECHO 1 strain.

The study of the specimens from herpangina cases produced striking results. Only from one did we succeed in isolating a "herpangina strain" (C A5), but it also contained the C B3 virus. The other 3 strains isolated proved to be identical with the latter.

Most of the positive samples originating from patients with febrile disease involving uncertain clinical symptoms and CNS lesions likewise yielded C B3 virus. This same strain was recovered from the CSF of the 3 cases of aseptic meningitis. In 2 cases of aseptic meningitis the same faecal sample harboured two different kinds of virus. Especially interesting was the one from which along with a C B3 strain a poliovirus type 1 strain was isolated, since the two viruses are known to interfere with each other in tissue cultures [15]. The other of poliovirus type 1 and 2 strains isolated occurred in specimens from patients afflicted with typical poliomyelitis.

After the epidemic 304 specimens originating from 209 individuals were tested for the presence of enteroviruses (*Table II/b*) and yielded 35 positive findings, C B2 and 3, ECHO 9 and 14, poliovirus type 1, and several as yet unidentified strains. None of the available type sera (see *Materials and methods*) neutralized the latter. Most of the strains were obtained from faeces, 2 from CSF, and 1 from throat swab. All 6 C B2 strains isolated after the epidemic were found in specimens from patients of the Poliomyelitis Rehabilitation Hospital, Budapest, during a hospital outbreak of "3-day fever".

Geographical distribution of virus-positive individuals. The majority of the specimens originated from persons living in the western parts of the country, or residing in Budapest. The positive cases came predominantly from these areas, as illustrated in *Fig. 1*. C B3 strains, however, were isolated in every part of the country.

Isolations of C B3 strains grown with two different methods. The isolated C B3 strains were biologically different. Some of them could be isolated only in monkey kidney tissue cultures, others only in suckling mice. *Table III* shows that of the 261 strains obtained 102 were isolated by the suckling mice technique, 129 by the use of monkey-kidney tissue cultures, while 30 by both

Table III

Isolations of Coxsackie B3 strains by means of two different methods

Disease group	Specimen	M. K. + S. M. +	M. K. + S. M. —	M. K. — S. M. +	M. K. + S. M. ∅	M. K. ∅ S. M. +	Total
Newborns and their environmental contacts (II—III)	Stools	12	1	11	9	—	33
	CSF	3	—	1	—	—	4
	Miscell.	10	—	2	3	1	16
All other groups (I, IV—VII)	Stools	4	12	11	100	74	201
	CSF	1	—	—	2	—	3
	Miscell.	—	1	—	1	2	4
Total		30	14	25	115	77	261

M. K. = monkey-kidney tissue culture

S. M. = suckling mice

+ = positive

— = negative

∅ = not employed

methods. Twenty-five of the specimens yielding virus when tested in suckling mice proved negative in monkey-kidney tissue culture, and 14 of those positive in tissue culture failed to yield virus when tested in baby mice. In the newborns involved in the two hospital outbreaks that biological variant was more frequent which could be isolated in suckling mice only, while in the other disease groups the two variants occurred in about the same proportion. It needs to be mentioned, however, that in the latter groups our tests failed to establish the true frequency rates of the individual biological variants because from most of the positive specimens we had attempted isolation with one method only.

Table IV

Isolation of Coxsackie B3 strains from faecal samples collected at varying times after onset of Bornholm disease

Number of days following onset	Number of samples tested	Number of Coxsackie B3 positives
1—5	166	119
6—10	51	31
11—15	17	8
16—30	16	5
>31	3	1
unknown	10	2
Total	263	166

Isolation rates of C B3 strains obtained from faeces collected at different points of time after onset of Bornholm disease. Table IV shows that more faecal samples were collected in the first five-day period of the disease than in all the subsequent periods. It was in this period that, with 72 per cent, the isolation rate was the highest, whereafter it gradually decreased to 61, 47, and 31 per cent. One of three specimens was still found positive as late as 30 days following onset.

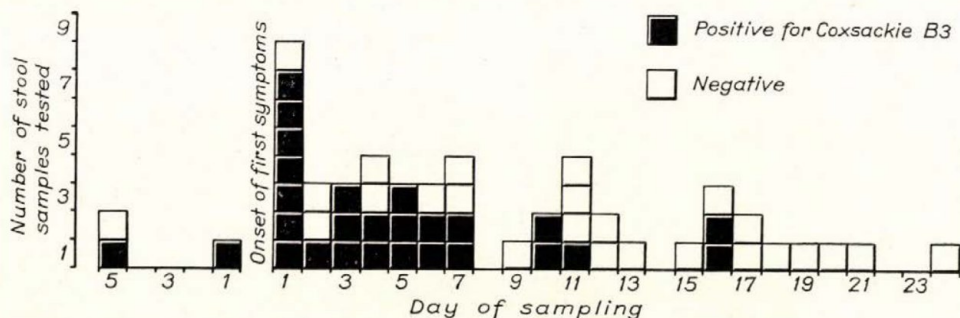


Fig. 2. Number of successful isolations from newborns' faecal samples collected at various days before and after onset of first symptoms

Isolation rates of C B3 strains from the faeces of diseased newborns, collected at different points of time after onset. From the majority of the newborns several samples were examined. Fig. 2 shows the number of isolations achieved from faecal samples taken on different days after the onset of symptoms. From three newborns samples were available before the first symptoms had appeared. One of the two faecal samples taken 5 days before the disease had become apparent already contained the pathogenic agent. The other was negative, yet the baby from whom it had originated, died 4 days after onset of the disease, and all the organs examined post mortem contained the C B3 virus. The faeces of a newborn collected one day before the first symptoms had appeared likewise proved positive.

The faeces of the newborns were positive up to 16 days after onset of the disease. In this group too, the isolation rate was the highest during the first week, to decrease gradually thereafter.

Age distribution of patients tested. This is shown in Table V, which however does not include the newborns and their contacts. Of all the patients, 74 per cent were below 20 years of age. This proportion holds good for the individual disease groups as well. In the group of Bornholm patients 66 per cent were under 20 years of age. The oldest person to suffer from Bornholm disease was 67 years of age. The distribution by sex of Bornholm patients and affected newborns showed no characteristics.

Table V
Age distribution of patients examined

Disease group	Age in years					
	0—10	11—20	21—40	41—	unknown	total
I. Bornholm disease	95	77	66	16	7	261
IV. Febrile states	54	14	7	0	4	79
V. Herpangina	16	2	0	0	1	19
VI. Lesions of central nervous system	108	50	29	12	8	207
VII. Miscellaneous conditions	58	17	12	7	6	100
Total	331	160	114	35	26	666
%	50	24	17	5	4	100

Table VI
Distribution of isolations by age

Age, years	Group of Bornholm patients					All other disease groups together				
	Number of persons tested	Positive for				Number of persons tested	Positive for			
		C B3		other viruses			C B3*		other viruses	
		number	%	number	%		number	%	number	%
0—10	95	77	81	4	4	236	22	9	26	11
11—20	77	54	70	1	1	83	10	12	4	5
21—40	66	28	42	—	—	48	5	10	—	—
41—	16	5	31	1	6	19	2	10	—	—
unknown	7	5	.	—	—	19	1	.	—	—
Total	261	169	65	6	2	405	40	10	30	7

* This column includes isolations of C B3 concurrently with other enteroviruses

Age distribution of total number of positives. Table VI shows the successful isolations by age groups of Bornholm patients as compared with those of all the other disease groups put together. In the Bornholm group the isolation rate of C B3 strains decreased with advancing age from 81 to 31 per cent, but in all the other disease groups it was uniformly low, around 10 per cent. With the exception of one, all the viruses other than C B3 were recovered from individuals under 20 years of age.

Testing sera for neutralizing antibodies against C B3 virus. For C B3 neutralizing antibodies the sera of 300 individuals were tested, of whom 151 belonged in Group I and 149 in Groups IV—VII. From 113 patients of Group I

Table VII

Antibodies to Coxsackie B3 in the sera of patients grouped according to virus isolation results

	Group I: Bornholm patients					Groups IV—VII: all other patients				
	positive for		Nega- tive	Total	%	positive for		Nega- tive	Total	%
	Coxs. B3	other viri- ses				Coxs. B3	other viri- ses			
Number of <i>titrated</i> paired sera	79	3	31	113	100	11	9	106	126	100
convalescent sera	21	2	15	38		—	2	21	23	
Number of <i>positive</i> * paired sera***	18	1	14	33	86	2	—	10	12	19
convalescent sera	20	2	8	30		—	1	4	5	
<i>Titre rise</i> in paired sera	58	1	8	67		4	1	7	12	
Number of <i>negative</i> ** paired sera	3	1	9	13	14	5	8	89	102	81
convalescent sera	1	—	7	8		—	1	17	18	

* Serum titres $\geq 1:5$ ** Serum titres $< 1:5$

*** Serum titres identical

paired sera were examined, and from 38 patients convalescent sera only. From Groups IV—VII, 126 paired sera and 23 convalescent sera were tested. Serum was usually collected in the first week and convalescent serum in the third week after onset. The results are listed in *Table VII*. No serum was regarded as positive unless it neutralized C B3 in titres of 1:5 or higher, and no paired sera were considered positive unless the acute and the convalescent components gave the same antibody titre.

The sera from 86 per cent of the Bornholm patients, but only from 19 per cent of the subjects afflicted with other diseases, were found to contain C B3 neutralizing antibody. A rise in titre was established in the majority (59 per cent) of the paired sera from Bornholm patients. The proportion of the serologically positive subjects (96 per cent) and of the paired sera with a rise in titre (73 per cent) was the highest among individuals who excreted C B3 virus. The proportion of serological positivity was also high (67 per cent) among the Bornholm patients who failed to yield a virus or yielded a virus other than C B3; moreover, in 24 per cent of the latter a rise in titre was observed.

In the other disease group (IV—VII), only the C B3 excretors yielded a fairly high proportion of serologically positives, though even there, in a dilution of 1:5, the blood of almost half of the patients contained no C B3 antibody.

As regards these latter subjects, it seems improbable that the B3 strains isolated from them should have been the pathogenic agent actually responsible for their respective disease. On the other hand, the serological results obtained make it apparent that in addition to being the causal virus of typical Bornholm disease, C B3 virus may have been the one to give rise to several other conditions listed with Groups IV—VII. At any rate, account must be taken of the possibility that some of the patients placed in these groups were positive for C B3 or showed a rise in titre, because they had passed through a latent infection with the strain.

Antibody titres above 1 : 85 were seldom obtained in Bornholm patients. The most frequently occurring antibody titre was 1 : 24, observed in the third week after the onset. The results for the sera from the newborns and mothers (Groups II and III) have not been included in the data in *Table VII*, since they have been published in detail elsewhere [4].

Comparison of clinical and aetiological findings in Bornholm patients. In Bornholm disease the clinical picture is known to vary according to the region of the body in which muscular pains are felt. The data submitted to this Institute concerning symptoms of 152 Bornholm patients excreting C B3 virus allowed us to study the frequency of the principal disease forms in the various age group.

Table VIII

Frequency of the principal disease forms in various age groups of Bornholm patients excreting Coxsackie B3 virus

Age in years	Disease forms				Total
	Abdominal	Thoracic	Abd. and thoracic	Others	
0—5	29	2	2	5	38
6—10	17	9	2	5	33
0—10 together	46	11	4	10	71
%	65	15	6	14	100
11—15	8	14	4	1	27
16—20	3	15	4	1	23
>20	4	22	4	1	31
>10 together	15	51	12	3	81
%	18	63	15	4	100
Total	61	62	16	13	152
%	40	41	10	9	100

Table VIII illustrates that in the age group under 10 years of age the form involving abdominal signs, and in all the other age groups that involving thoracic symptoms, was preponderant. The abdominal form was observed in 65 per cent of the patients under 10 years of age, and the thoracic in 15 per cent only, whereas of the subjects above the age of 10 years, only 18 per cent suffered from the abdominal, and as much as 63 per cent from the thoracic form.

Table IX

Frequency of associated meningitis and herpangina in Bornholm patients

Diagnosis	Number of patients studied	Results of isolation		
		positive for C B3	positive for other viruses	negative
Bornholm disease	213	134	5	74
Bornholm + meningitis ..	12	7	0	5
Bornholm + herpangina ..	36	28	1*	7
Total	261	169	6	86

The clinical pattern of Bornholm disease may also vary in dependence on the eventual complications. *Table IX* reveals that in association with Bornholm disease meningitis occurred in 4.6 per cent, herpangina in 13.8 per cent, of the 261 cases studied. C B3 strains were isolated from 7 of the 12 Bornholm cases complicated with meningitis — a syndrome called meningitis myalgica by GSELL [16] —, and from 28 of the 36 patients with associated herpangina. Not one of these 36 yielded C A virus, although the specimens of 30 of them had been tested in suckling mice. Eight of the 12 Bornholm patients with meningeal symptoms and as many as 23 of those with herpangina were less than 10 years of age.

The white blood cell count was determined in 67, the erythrocyte sedimentation rate in 70 Bornholm patients. The white cell count was normal (< 10 000) in 78 per cent and elevated in 22 per cent of the cases. ESR was less than 10 mm/hr in 40 per cent and more than 20 mm/hr in 34 per cent of the patients examined.

Discussion

We have no proof of Bornholm disease having ever occurred in Hungary until 1958. On the other hand, we have good reason to believe that the outbreaks of "appendicitis" recorded by PETRILLA [17] in 1938 and JÓSA [18] in 1939 were actually Bornholm epidemics.

The vast proportions of the 1958 epidemic and the fact that, though not forming the majority, persons in the older age groups were involved in it

* C B4 strain

great numbers, seem to favour the inference that C B3 strains had not been occurring in Hungary for a long time prior to 1958. In 1950 and 1951, isolations of this viral type were reported from many parts of the world but in Hungary — where, it is true, systematic Coxsackie research work had not yet been in progress — there were no epidemiological signs to point to C B virus infections. In this country C viruses were for the first time isolated in 1952, and all of those isolated until 1958 were C A strains [12, 19–22]. One of us [12] has reported the results of Coxsackie investigations carried out in this Institute up to 1954. Here we would only add that each year from 1954 to 1958 are worked up from 250 to 300 specimens from patients suspect of various infections and could regularly isolate C A viruses in 4 to 5 per cent of them. Under these circumstances it is a point of special interest that in 1958 there occurred B1, B2, and B4 type strains in addition to the B3 type strain responsible for the epidemic.

The results of our investigations leave little doubt that the country-wide Bornholm epidemic of 1958 and the two hospital outbreaks among newborns described in this paper, were caused by the C B3 virus. It was this type we isolated from 65 per cent of the Bornholm patients, and antibody to this type was found in the blood of 86 per cent of them after recovery. A similarly high — 62 per cent — recovery rate was encountered in the affected newborns. On the other hand, only 13 per cent of the patients suffering from other diseases, yielded C B3 virus and the blood of only 19 per cent contained antibody to it. Our findings make it appear probable that in some of the cases in these groups, particularly in those with febrile episodes and aseptic meningitis, the C B3 virus was the pathogenic agent. The successful isolations of virus from CSF together with the fact that 4.6 per cent of the Bornholm patients developed meningitis as a complication furnish evidence that C B3 virus was responsible for some of the cases displaying symptoms of aseptic meningitis. The close relationship between Bornholm disease and aseptic meningitis had been emphasized by many authors long before the aetiology became known, and has since found confirmation from several quarters [23]. Thus, in this respect too, our findings are in agreement with those of earlier workers.

It is remarkable, on the other hand, that with the exception of two patients, we failed to recover virus other than C B3 from the cases diagnosed clinically as herpangina or herpangina combined with Bornholm disease. The current view links herpangina with certain C A strains [24], and we know of only one case in the literature incriminating a B3 strain as the cause of a herpangina-like syndrome combined with aseptic meningitis and pleurodynia [25]. On the evidence of our findings it seems more probable that C B3 virus was responsible for the symptoms of herpangina in our positive cases.

In isolating C B3 strains the tissue culture procedure is now everywhere given preference to the suckling-mouse technique. According to our experi-

ence, however, the use of only one of these methods is not satisfactory, there being strains which cannot be recovered by means of the one procedure but are readily isolated with the other. In respect of C B3 strains, a similar conclusion has been reached by HABEL *et al.* [26]. Our results indicate that for frequency of occurrence the two biological variants may depend on the age of the individual in whom they grow, considering that with one single exception, from newborns we could isolate solely "suckling mice" variants.

Numerous authors found that children as well as adults might excrete C viruses with the faeces for a long time after recovery. Our own findings have supported this observations, yet showed that lasting excretion of the virus is less frequent in newborns. Sixteen days after onset was the longest period of time during which C B3 virus could be isolated from the faeces of newborns.

On the strength of epidemiological studies and animal experiments, DALLDORF [7, 27–29] was the first to postulate an antagonism between C B and polioviruses. The country-wide Bornholm epidemic under review has produced evidence suggesting that these two viral infections tend to be mutually exclusive. In 1958, the year of the great Bornholm epidemic, the incidence of poliomyelitis in the whole country was the lowest observed over a 20-year period. There occurred as few as 165 cases of poliomyelitis during the whole year (1.7 per 100 000). One should think a sufficiently plausible explanation for this is the immunity which might have been imparted to large portions of the population that had gone through a latent stage of the infection during the preceding year or was brought forth by the mass Salk vaccinations initiated in August 1957. There are, however, considerations which seem to contradict such a view.

a) In the years preceding 1958 the incidence of poliomyelitis was always lower in Transdanubia than in the eastern part of the country. For the years 1950–57 the average morbidity rate was 6.2 per 100 000 inhabitants in the western as against 11.0 in the eastern region. In 1957, when the country experienced its until now most vehement outbreak (2334 cases) with poliovirus type 1 as the causative agent [30, 31], the morbidity rate still remained low in Transdanubia. Nevertheless, in 1958 the incidence of poliomyelitis was slightly lower in Transdanubia (1.4 per 100 000) than in those parts of the country (1.6 per 100 000) which having been severely affected by the 1957 epidemic, had better chances to acquire immunity.

b) Another characteristic of the year 1958 was that the poliomyelitis epidemic curve did not show the usual summer peak. In Hungary in the years 1950–57, 55.0, 61.1, 71.6, 53.1, 52.0, 48.0, 53.9 and 62.5 per cent, respectively, of the poliomyelitis cases occurred in the third quarter of the year. In 1958, on the other hand, only 28.4 per cent were reported from the third quarter and the distribution of cases was equal in the rest of the year. (Percentual distribution, 24.3, 27.9 and 19.4 per cent, respectively.) The thought may arise

that the unusually low incidence in the third quarter of 1958 was due to the booster dose of Salk vaccine given in the spring as the third injection of the mass vaccination begun in 1957. What makes this seem improbable are our observations made in 1959. In this year in spite of the mass vaccinations a poliomyelitis epidemic nearly as severe as that in 1957 was experienced (1830 cases) and 75.9 per cent of the cases occurred in the third quarter of the year.

It can therefore be concluded that the unusually low incidence of poliomyelitis in 1958, and particularly in the third quarter of the year, was due to the Bornholm epidemic which had its peak in that quarter.

Our findings confirm DALLDORF's view on the antagonism of C B and polioviruses in nature, and his conclusion that the low morbidity and mortality rates of poliomyelitis in Sweden in the year 1931, may be ascribed to the simultaneous Bornholm epidemic [29].

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Address of the authors:

ISTVÁN DÖMÖK, ERZSÉBET MOLNÁR, OTTÓ RUDNAI
State Institute of Hygiene, Gyáli ut 2/6, Budapest IX., Hungary

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SABIN, A. B. (University of Cincinnati College of Medicine, Cincinnati, U. S. A.): *Status of field trials with an oral, live attenuated poliovirus vaccine.*

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CHUMAKOV, M. P., VOROSHILOVA, N. K., VASILEVA, K. A., BAKINA, M. N., ASHMARINA, E. E., DOBROVA, I. N., DROZDOV, S. G., YANKEVICH, O. D., PODSEDLOVSKY, T. S., SOKOLOVA, I. S., SHIRMAN, G. A., BOYKO, V. M. (Polio-myelitis Research Institute of the Soviet Academy of Medical Sciences, Moscow, USSR): *Large-scale oral immunization in the USSR with a live vaccine prepared from Sabin's attenuated poliovirus strains.*

Serial production of the highly immunogenic live poliomyelitis vaccine prepared from SABIN's attenuated strains has been started in the USSR. The vaccine which in its basic properties agrees with SABIN's original vaccine is administered in the form of dragée-candy. Its safety, previously proved by ŠKOVŘÁNEK, ŽÁČEK, SMORODINTSEV, HALE and others, has been definitely established by the mass vaccination carried out in the USSR. In the Estonian and Lithuanian Republics, where vaccination had almost been completed by June 1, 1959, a substantial decline in poliomyelitis morbidity was observed during the following summer (sporadic cases were reported only). This might be regarded as the first evidence of the epidemiological efficacy of the vaccine. The campaign with the live vaccine carried out in Tashkent in the course of the 1959 epidemic has confirmed the view that poliomyelitis morbidity substantially is reduced within 3 or 4 weeks probably by the interference existing between the vaccine strain and wild virus, on the one hand, and by the gradual immunobiological conversion of susceptible subjects, on the other. It seems possible to eradicate poliomyelitis in the USSR within one or two years by immunizing the whole population with the live vaccine.

ŠKOVŘÁNEK, V. (Ministry of Health, Prague, Czechoslovakia): *Field trial with the Sabin-type live attenuated poliomyelitis vaccine in Czechoslovakia.*

In Czechoslovakia the first European large-scale field trial with SABIN's live poliovirus vaccine was carried out in the winter 1958—1959. Over 100 000 children aged from 2 to 6 years were treated by mouth by the three types of attenuated virus at intervals of one month. The order of administration was type 1, type 3, and type 2. Both before and after vaccination an extensive serological survey and in certain communities of young children, detailed studies were done. Prior to the vaccination, in spite of the preceding large-scale immunization with the Salk vaccine, in children from 2 to 8 years of age the incidence of antibodies to type 1 virus was as low as 65.5 per cent; that to type 3, 55.8 per cent. As a result of the immunization with the live vaccine 94.7 per cent became positive of the 38 children who had had no antibodies to type 1. The percentage of the converters for types 2 and 3 were 83.3 and 85.0, respectively. About 50 per cent of the unvaccinated contacts showed a significant rise in their neutralizing antibody titres. No rise in the poliomyelitis morbidity rate or other untoward effects were observed during the field trial. Since then the morbidity rate for the areas involved in the campaign has been lower than for those where Salk vaccine had been administered instead of the live vaccine.

FARKAS, E. (State Institute of Hygiene, Budapest, Hungary): *Present status of the poliomyelitis problem in Hungary.*

A brief account has been given of the virological and epidemiological research into poliomyelitis carried out in Hungary. Most of the papers cited have been published in this Journal [PINTÉR, M. *et al.* 3, 399 (1956), DÖMÖK, I.: 4, 183 (1957). FÖLDES, P.: 4, 197 (1957), MOLNÁR, E. and FORNOSI, F.: 4, 353 (1957), DÖMÖK, I.: 5, 111 (1958), PETRILLA, A.: 5, 297 (1958), RUDNAI, O.: 5, 359 (1958), MOLNÁR, E.: 5, 369 (1958), FÖLDES, P. *et al.*: 6, 257 (1959).].

The 1957 epidemic, which had affected about a third of the country's territory had not spread over the unaffected areas in 1958 but did so in 1959. There is little doubt that the Bornholm epidemic caused by the Coxsackie B3 virus, which in 1958 had spread practically over the whole country, interfered with the spread of poliomyelitis (see p. 151). In 1958 intramuscular Salk vaccination was made compulsory for infants, and available free of charge for every person under 18 years of age. In the summer of 1959 the age group under 10 years received a second booster dose (*i. e.* fourth dose) of Salk vaccine. In spite of this about 1600 cases of paralytic poliomyelitis were reported by mid-September of the same year. According to preliminary evaluation this number would have been substantially higher without Salk vaccination. Out of the 448 polio-

virus strains isolated and identified in 1959, 443 proved to belong to type 1. Hungarian-made Salk vaccine (*State Institute of Hygiene, Budapest*) was first used for mass vaccination in the summer of 1959.

WAGENER, K. (Institute for Veterinary Hygiene, Veterinary High School, Hannover, Germany): *Influence of antibiotics on viral infections.*

Since the end of World-War II certain viral diseases often show a milder clinical course, making diagnosis difficult. The author supposed that serial natural passages in animals fed with antibiotics might have attenuated the viruses. In his wide-scale experiments, however, the clinical course of swine fever in aureomycin-fed animals hardly differed from that in the control pigs. Survival times were somewhat longer in the experimental groups but did not tend to lengthen during the ten passages made in aureomycin-fed animals. Thus the slight difference in the survival times may be explained by the effect of the antibiotic on enteric flora. Similar experiments, essentially with the same results, were carried out with NDV in chickens fed with terramycin.

VOROSHILOVA, M. K. (Poliomyelitis Research Institute of the Soviet Academy of Medical Sciences, Moscow, USSR): *Enteric non-poliomyelitis virus infections in the USSR.*

In the USSR enteroviruses have been investigated since 1946. Viruses have been isolated in monkeys, cotton rats, newborn white mice, human embryonic tissue cultures, HeLa cells and monkey-kidney cell monolayers. In 1952 three peculiar strains of enterovirus were isolated from paralytic cases in connection with a poliomyelitis epidemic affecting 260 persons in Karaganda. Two of the cases yielding virus were fatal in outcome. Swedish and USA authors have found a close relation between a representative (AB-IV) of these strains and the prototype of the Coxsackie A7 virus. The high neuropathogenicity for monkeys of the AB-IV strain has been proved in several laboratories. (One case of paralytic poliomyelitis due to Coxsackie A7 virus was reported later by American authors.)

The authors have demonstrated that the prototype strain of Coxsackie A7 virus is also paralytogenic for monkeys. Neutralizing antibodies to the AB-IV strain are very common among the population of the USSR. In the age groups under 1 year, 1-6 years, 6-16 years, and over 16 years antibody was demonstrated in 5%, 32%, 53% and 80%, respectively, of the serum samples tested. Of the immunological types of ECHO virus types 1, 4, 6, 7, 8, 9, 11, 13 and 19 have been isolated in the USSR. An epidemic of aseptic meningitis with unusually high morbidity rate among children under 10 years of age was observed in the Far East regions of the USSR in 1958. Over 150 strains including polio type 2, ECHO-1 and ECHO-13 were isolated from faecal samples

taken from patients affected by the epidemic. In the gamma globulin prepared in the USSR neutralizing antibodies to all the ECHO and cytopathogenic Coxsackie types are present.

FORNOSI, F. and HORVÁTH, S. L. (State Institute of Hygiene, Budapest, Hungary): *Spreading of type 1 poliovirus among contacts of acute patients in a children's home.*

To be published in full in this Journal.

KARASSZON, D. (State Institute of Hygiene, Budapest, Hungary): *Histopathology of biologically different poliovirus type 1 infections.*

A total of 22 rhesus monkeys was infected intraspinally with the following strains of type 1 poliovirus, Mahoney original, Mahoney Cincinnati, LLc 2 ab, Csopak 15 (isolated from a child with paralytic poliomyelitis), and Csopak 157 (isolated from a healthy contact of the child excreting the strain Csopak 15). Marked differences were observed in the pathogenicity of the strains. Even the two strains obtained from the same children's home were different in pathogenicity.

DÖMÖK, I. (State Institute of Hygiene, Budapest, Hungary): *Multiplication of poliomyelitis virus in the central nervous system of young mice having survived a Coxsackie B1 virus infection.*

Published in full in this Journal (7, 87, 1960).

DÖMÖK, I. and MOLNÁR, E. (State Institute of Hygiene, Budapest, Hungary): *Virological examination of sporadic abacterial meningitis.*

In 1958 and in the first 6 months of 1959, 244 faecal specimens and 184 CSF specimens from 253 patients with aseptic meningitis were examined in monkey kidney tissue cultures and in suckling mice. Virus was isolated from 40 specimens of 36 patients. Of the 38 strains recovered from faecal specimens 1 Coxsackie (C) A5, 1 C B2, 19 C B3, 1 ECHO 4, 3 ECHO 9, 2 ECHO 14 and 1 polio-1 strains were identified. The identification of 6 strains is in progress. Two faecal specimens yielded 2 virus strains each, poliovirus 1 + C B3, and C A2 + C B3, respectively. Four strains, viz. 2 C B3, 1 ECHO 9 and 1 unidentified strain, were isolated from CSF specimens. The ratio of successful isolation was 28 per cent during the summer months and only 9 per cent in the rest of the year. CF tests performed in paired sera of 161 patients supported the causative role of mumps virus in 43 cases most of which had occurred in the winter periods.

FÖLDES, P., SZERI, I. and BÁNOS, Zs. (Institute of Microbiology, University Medical School, Budapest, Hungary): *Hungarian and foreign poliomyelitis vaccine preparations: Their antigenicity and thermal sensitivity on storage.*

To be published in full in this Journal.

ZHDANOV, V. M., FADEEVA, L. L. (Ivanovsky Institute for Virology, Soviet Academy of Medical Sciences, Moscow, USSR): *Studies on the live measles vaccine.*

(i) The biological properties of measles virus grown in tissue cultures were investigated. The 3 strains involved in the experiments had been isolated in the USSR, England and USA, respectively. The strains grew well in trypsinized cell cultures of both monkey kidney and human amnion and evoked the same cytopathic changes and about equally high titres of complement fixing antigen in tissue cultures.

(ii) The immunogenic effect in monkeys of the tissue-cultivated strains has proven satisfactory.

(iii) Measles vaccine was prepared in the allantoic cavity of the chicken embryo from strains which had been cultivated in human embryonic lung tissue cultures. Children were three times inoculated with the vaccine intranasally or percutaneously. An immunity comparable to that following natural infection could not be observed in the field trials.

(iv) Measles vaccines prepared from pure lines of tissue-cultivated strains were applied in children's communities. According to preliminary data based on the immunogenicity of the vaccines and the mildness of the untoward reactions, this type of vaccine is expected to be generally applicable but a higher concentration of virus and an improved schedule of vaccination are needed.

NIKOLIC, M. (Pasteur Institute, Novi Sad, Yugoslavia): *On the relation of a bat virus to human encephalomyelitis.*

Bat viruses from Yugoslavia and Turkey, encephalomyelitis viruses from sporadic human cases from the USSR, and the fixed virus of rabies were compared in laboratory animals. The bat virus from Yugoslavia and the human strains from the USSR were found to be identical with each other but in many characteristics different from the street virus. The bat strain from Turkey and the street virus, though exhibiting many similarities, could not be regarded as identical.

The possible aetiology of certain human encephalomyelitides and ascending myelitides has been discussed in relation to rabies virus and the agent isolated from bats. The existence of a pure "paralytic rabies" is questionable.

BÁRDOS, V. (Institute of Epidemiology and Microbiology, Bratislava, Czechoslovakia): *Properties and ecology of a virus isolated from mosquitoes in Czechoslovakia.*

Five strains of a neurotropic virus were isolated from mosquitoes in the summer of 1958, three of them from *Aedes vexans* and two from *A. caspius*. The isolated virus was given the name "Tahyna".

The results of the virological and serological tests made in order to identify the virus have been presented. Serological studies revealed a high incidence of virus neutralizing antibodies in sera of human beings and domestic animals in localities infested every year by mosquitoes.

In areas irregularly invaded by mosquitoes the antibody was found less frequently; it was absolutely absent in regions without mosquitoes. A serological study of birds from the localities where the virus had been isolated indicated that birds may be of some ecological importance of the virus Tahyna in nature.

GREŠÍKOVÁ, M. and ŘEHÁČEK, J. (Institute for Virology, Czechoslovak Academy of Sciences, Bratislava, Czechoslovakia): *Isolation of tick encephalitis virus from the blood and milk of domestic animals (sheep and cattle) infested by Ixodes ricinus L.*

On subcutaneous inoculation of tick-borne encephalitis virus the excretion of small quantities of the agent into the milk of cows and goats was observed.

The transfer of tick-borne encephalitis virus by infected ticks to sheep and a cow has been studied. Sheep were found to excrete in their milk the virus of louping ill on the 7th and 8th days, while tick-borne encephalitis virus from the 3rd to the 7th days, after infection. From blood samples taken from the infected cow the virus was recovered on the 2nd and 6th days, while from the milk on the 3rd and 4th days after infection.

The epidemiological relations of these findings have been discussed.

LIBÍKOVÁ, H. and VILČEK, J. (Institute for Virology, Czechoslovak Academy of Sciences, Bratislava, Czechoslovakia): *Laboratory diagnosis of tick encephalitis in HeLa cell cultures.*

Under modified circumstances of cultivation HeLa cells were found to exhibit cytopathic changes when infected with tick-borne encephalitis virus. The sensitivity of HeLa cells to the mouse-adapted virus was remarkably lower than that of the mouse to either intracerebral or intraperitoneal infection. On repeated passages in HeLa cultures infectivity was found to increase and after a certain number of passages it reached cytopathogenic titres equal to

those usually obtained in mice. Multiplication of virus resulted in the inhibition of cell metabolism, thus making the development of a colour test possible.

Both cytopathogenicity and colour test were used for the demonstration of virus and neutralizing antibodies.

BORECKÝ, L. and ZÁVADA, J. (Institute for Virology, Czechoslovak Academy of Sciences, Bratislava, Czechoslovakia): *The effect of anticellular sera on the multiplication of Myxoviruses.*

In a certain period of its multiplication the virus becomes an integrated part of the infected cell. Specific anticellular, or anticell-organell sera might accordingly influence the multiplication of the virus inside the cell.

The first part of a study concerning this problem has been presented, using NDV-infected chorio-allantoic and/or fibroblast cells and sera collected after immunization of rabbits and guinea pigs with different chick embryonic tissues as a model system. The antichorioallantoic, anti-chick-embryonic-extract and antichick-fibroblast sera were found to exert a marked influence on NDV multiplication independently of the time when the sera had been added to the cells. The findings suggested a direct effect of the sera on the cell studied.

Surprisingly, some sera from normal guinea-pigs, rabbits and rats were found to contain active inhibitors of the intracellular multiplication of the virus. These inhibitors were destroyed by heat. The mechanism of virus inhibition of normal active sera appeared to be different from that of anti-cell immune sera.

BÉLÁDI, ILONA and KUKÁN, ESTHER (Institute of Microbiology, University Medical School, Szeged, Hungary): *Virus isolation and antibody demonstration experiments with haemadsorption viruses.*

From the throat washings of 100 children suffering from different diseases of the upper respiratory tract no haemadsorption virus could be isolated. The 25 other agents isolated in HeLa cultures produced cytopathic changes mainly characteristic of adenoviruses. Out of them 11 were identified as adenovirus by the complement fixation test. From further 100 pharyngeal washings up to now one haemadsorption virus has been isolated. This haemadsorption virus forms syncytium in HeLa cells. The complement fixation test carried out with the paired sera from the patient from whom the virus was isolated revealed antibody in the titre of 1 to 64, in the convalescent specimen while none in the acute serum.

A total of 140 human sera was tested against the haemadsorption virus type 1. Neutralizing antibody titres of 1 to 128 or higher were found in 23 per cent.

NÁSZ, I. and TÓTH, M. (Institute of Microbiology, University Medical School, Budapest, Hungary): *Complement fixing and neutralizing antibodies to adenoviruses in various groups of the population of Hungary.*

Elevenhundred and seventy-four human sera were examined by the complement fixation (CF) and 148 sera by the neutralization test for antibodies to adenoviruses. The sera examined for CF antibodies originated from 6 groups, (i) 85 children, most of them under 5 years of age, recovered from poliomyelitis; (ii) 61 tuberculosis patients, 10–20 years of age; (iii) 163 healthy adolescents, 18–20 years of age; (iv) 117 healthy adults; (v) 632 patients with non-respiratory disease; (vi) 116 patients with acute respiratory disease. The percentage of positive sera in groups (i)–(vi) were 48.2, 24.5, 14.7, 13.6, 42.4 and 48.2; the average titres, 1 : 8.8, 1 : 11.6, 1 : 4.8, 1 : 10.7, 1 : 5.6 and 1 : 7.3, respectively. The highest titre in each group was 1 : 64; titres 1 : 4 and 1 : 8 were the most frequent. The incidence of positive sera was somewhat lower in subjects over 40 or 50 years of age than in the younger age group. The 148 sera were tested for neutralizing antibodies against the first 7 types of adenoviruses. The incidence of type 1, 2 and 7 antibodies was higher than that of the others.

SCHMELZER, K. (Institute for Phytopathology, German Academy of Agricultural Sciences, Aschersleben, Germany): *The application of tissue cultures in plant virology.*

(i) By means of meristem culture the elimination of potato virus Y from the potato variety "Carla" has been successful. This variety was completely infected with virus Y.

(ii) Tissue cultures prepared from tobacco stem could be infected with tobacco mosaic virus by using the pin puncture method. The percentage of infection varied greatly with different plant clones.

(ii) By continuous selection on the tissue clones "Y II" artificially infected with tobacco mosaic virus, totally infected lines were obtained from the fifth passage on. In the case of the clone "sain", 15 per cent of the secondary cultures proved to contain virus. Nevertheless, 20 per cent of the secondary cultures of "*Nicotiana glauca* × *N. langsdorffii*" were devoid of virus even after the sixth passage. Accordingly, the tissue clones are supposed to be accessible to systemic virus infection to a different extent, or they are able to eliminate tobacco mosaic virus only partially.

(iv) Addition of the growth substance naphthyl acetate to cultures infected with tobacco mosaic virus generally resulted in enhanced growth and a decrease of virus activity. The growth inhibitor malonic acid hydrazide acted in the opposite way. Individual plant clones reacted somewhat differently; the ability to synthesize auxine by the tissue cultures also impaired virus increase.

SOLYMOSY, F., FARKAS, G. L. and KIRÁLY, Z. (Research Institute for Plant Protection, Budapest, Hungary): *The mechanism of localization in plant virus infections.*

Virus infections eliciting only local necroses were compared with those causing a systemic disease. In the first case the penetration of virus was found to be followed by a sudden rise in the polyphenoloxydase activity while in the second the activation of this enzyme was slow. The high polyphenoloxidase activity was accompanied by a local accumulation of polyphenol oxydation products (quinones), demonstrable histochemically, and by a decrease in the polyphenol content.

Formation of the necrotic spots, which inhibit the spread of virus appears to be brought about by the accumulation of quinones, as lesion formation is inhibited by the application *in vivo* of quinone reducing substances, such as ascorbic acid, glutathion and cysteine. The quinone reducing substance content of plants which is sufficient to reduce the quinones in the healthy plant proved to be insufficient when the polyphenoloxidase activity rose high above normal. A decrease in ascorbic acid content parallel to the development of necrogenic reactions has been established.

BARTA, A. (Institute of Epizootology of the Veterinary College, Budapest, Hungary): *Virus adaptation experiments using combined cultures of cells from susceptible and insusceptible animals.*

The virus of the Teschen disease is pathogenic for hogs and does not multiply in tissue cultures from any other animal. To adapt the virus to cells of animals other than the combined cultures of horse and hog, resp. cat and hog kidney cells were inoculated. When carried through several passages the virus exhibited increasing affinity to horse kidney cells. Virus from the 20th passage on in combined cultures multiplied for 5 successive passages in pure horse kidney cell cultures, while under identical conditions the original strain died out in the 3rd passage.

SZENT IVÁNYI, T. (Institute of Epizootology of the Veterinary College, Budapest, Hungary): *Laboratory diagnosis of atypical Teschen disease and Aujeszky's disease by virus isolation on kidney cell monolayer.*

Successful isolation and identification of the virus of Teschen disease in hog kidney tissue culture was achieved from the pathomorphologically doubtful or negative brain of hogs exhibiting atypical neurological symptoms from two separate epizootic foci. The typical cytopathic changes were in some cases, observable only after one or two blind passages. The presence of Aujeszky's

virus in hog brains negative or non-typical on rabbit inoculation was demonstrated by hog kidney tissue culture inoculation. The typical cytopathic changes caused by Aujeszky's virus in tissue cultures made a tentative diagnosis possible before neutralization test could be carried out.

SZATHMÁRY, J. ("Human" Vaccine Production Research Institute, Budapest, Hungary): *Examination of nonspecific inhibitors of vaccinia virus haemagglutination.*

Independently of the presence of specific haemagglutination inhibiting antibodies in the blood, certain body fluids (CSF of high protein content, pleural, peritoneal, and hydrocele fluids, joint and ovarian cyst punctates) were found to have a nonspecific inhibitory effect on vaccinia virus haemagglutination.

The inhibitors are not dialyzable and appear to exert their effect directly on the haemagglutinin. No relation was found between the total protein and inhibitor content of the fluids. On salting out with ammonium sulphate the inhibitors were precipitated with the globulin fraction.

The mechanism of the non-specific-inhibitor — virus interaction seems to be very similar to that observed in the case of the specific inhibitors. The non-specific inhibitors are stable at 56° C, but partial inactivation takes place at 60° C. In contrast to this the specific antibodies present in the serum after vaccination are not impaired at 70° C. At 85° C all inhibitors are destroyed.

CSIKVÁRY, L., MARKOVITS, P. and TÓTH, B. ("Phylaxia" State Serum Institute, Budapest, Hungary): *Mass production of Newcastle disease virus in trypsinized chick embryo cell cultures.*

Mass production of a strain of ND virus isolated in Hungary was performed in trypsinized chicken embryonic tissue culture flasks. Most of the 14 lots prepared had infectivity titres between 10^{-7} and 10^{-9} . Virus multiplication appeared to take place in 6-hour cycles, reaching its peak at about the 20th hour after infection. A gradual decrease in infective virus content of the cultures was observed from the 2nd or 3rd day on. The haemagglutinating titre of the culture fluids attained its maximum at the 38th hour and exhibited no significant changes up to the 5th day. The possibility of using the tissue-cultured virus for immune serum production was also studied and the authors succeeded in producing immune sera against fowl plague as potent as the sera prepared with the virus produced in the chicken embryo.

SIMONYI, ERZSÉBET (State Institute for the Control of Veterinary Serobacteriological Products, Budapest, Hungary): *Titration of virus neutralizing sera in monolayer cell cultures.*

The neutralization test in monolayer tissue cultures was found to be an appropriate method for the titration of antibodies against any virus exerting a cytopathogenic effect. The method was used with success in the control of veterinary immune sera produced on a large-scale.

GRÉCZI, EMILIA, KOJNOK, J. and NÉMET, L. (Central Institute of Cancer Research, Budapest, and "Phylaxia" State Serum Institute, Budapest, Hungary): *In vitro oncolytic agent in the livers of dogs with canine hepatitis (Rubarth's disease).*

Extracts from the livers of dogs artificially infected with the virus of canine hepatitis and killed before death were found to have a damaging effect *in vitro* on "180" mouse sarcoma cells.

BLÁŠKOVIČ, D., SOKOL, F. and KOČIŠKOVÁ, D. (Institute for Virology, Czechoslovak Academy of Sciences, Bratislava, Czechoslovakia): *Characteristics of the soluble antigen and haemagglutinin of influenza viruses and some other myxoviruses.*

The method of LIEF and HENLE was successfully used for obtaining g-antigen (S-antigen) and haemagglutinin in several members of the myxovirus group (A influenza viruses, strains PR8, A2 Singapore, A anatis Kosice 1956, A swine — Iowa 15; fowl plague virus strain Dobson; NDV strain Blacksburg). The method was improved by high speed (15 000 r. p. m.) centrifugation for 45 min. to remove partially split and denatured virus. Ether treatment proved unsuitable with influenza virus A equi Praha 1956 because it caused denaturation of virus and loss of biological properties of its subunits.

Cross haemagglutination inhibition tests have shown that the haemagglutinins of the viruses tested are strictly specific. Cross complement-fixation tests indicated that according to their g-antigens, viruses A, A2, A anatis Kosice 1956, A swine and fowl plague belong to one group. Newcastle disease virus belongs to another group.

g-antigen of influenza virus A2 (Bratislava 4/1957) did not behave as an allergen, as tested intradermally in ferrets which had undergone infection with the same virus type.

Quantitative evaluation of the recovery of virus subunits after ether treatment by means of absorption spectrophotometry and nitrogen determination has shown that only 10—20% of virus originally present in the suspension was split. The separated subunits can be purified by high speed (40 000 r. p. m.) centrifugation for 120 min, but the maximum recovery rate was 50%.

HÁNA, L., KOČIŠKOVÁ, D. and STYK, B. (Institute for Virology, Czechoslovak Academy of Sciences, Bratislava, Czechoslovakia): *Characterization of an avid influenza A2 strain inhibitor.*

The avid A2 influenza virus haemagglutination inhibiting (HI) and neutralizing (VN) activity of protein fractions obtained by paper electrophoresis in alkaline and acid medium from sera of guinea pigs and horses was investigated. For guinea pig serum the maximum HI and VN activity at pH 9.0 was found to move together with the globulin fraction. At pH 4.6 the inhibiting activity moved faster toward the anode than the fractions of globulin and the albumins. This finding and the staining reaction of electrophoretograms for glycoproteins (Schiff) suggested a glycoprotein nature of the anti-A2 inhibitor.

STYK, B., KOČIŠKOVÁ, D. and BLÁŠKOVÍČ, D. (Institute for Virology, Czechoslovak Academy of Sciences, Bratislava, Czechoslovakia): *The immunological response to experimental infection with A2 influenza virus strains.*

Observations on the nature of the immunological response of white mice to infection with the Asian type (A2) of influenza virus are presented. Previous data on the thermolabile character of the antibody (or, more exactly, of the "antibody + cofactor complex") were confirmed by haemagglutination inhibition (HI) tests with the non-avid "A" influenza strains. However, the thermolability of this complex was found to vary with the development of immune reactions after both infection and active immunization. The specific antihaemagglutinins were thermolabile during the early stages of infection and after the first infection of antigen. After repeated injections of virus, the antibodies became resistant to heat.

Data presenting the effect of delayed reimmunization are presented together with preliminary results of experiments *in vivo* on the effect of increased body temperature. The latter appeared affect the titres of specific HI antibodies.

BARB, KATALIN and TAKÁTSY, GY. (State Institute of Hygiene, Budapest, Hungary): *Comparative studies on the antigenic structure of some influenza A2 virus strains.*

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TAKÁTSY, GY. and BARB, KATALIN (State Institute of Hygiene, Budapest, Hungary): *Recent studies on the erythrocyte receptor and non-specific serum inhibitor for the avid strains of influenza A2 virus.*

While all the influenza A2 strains isolated in Hungary in 1957 and 1958 belonged to the so-called avid variety, those isolated in 1959 proved to be non-avid or exhibited a wide-scale of avidity. The authors succeeded in isolating

a hyperavid and a non-avid component from an intermediate strain. The 1959 strains were variable in their affinity to erythrocytes, but all agreed in the capacity of being adsorbed to a receptor substance other than the mucoprotein receptor for the classical A strains. The non-specific inhibitor for the avid strains, though in many of its chemical characteristics similar to Francis inhibitor, was distinguishable from the latter by its different reaction with the avid strains.

TAKÁTSY, GY., BARB, KATALIN, and ANTONI, F. (State Institute of Hygiene, Budapest, and Institute of Biochemistry, University Medical School, Budapest, Hungary): *The characteristics distinguishing the incomplete form of influenza virus from the complete one.*

The density of the complete particles of influenza A virus was found to be 1.104, that of the incomplete ones 1.073 as determined in the analytical ultracentrifuge by the method of SHARP *et al.* Incomplete particles agree with the complete ones in their antigenic structure and diameter, but their specific water content and partial specific volume are different. Preliminary data suggest a heterogeneity of the nucleic acid composition of the complete particles.

HOLLÓS, I. (State Institute of Hygiene, Budapest, Hungary): *Inhibition of the multiplication of influenza virus by a modified Francis-inhibitor.*

To be published in full in this Journal.

NASTAC, E. and FUHRER, B. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Effects of certain viruses on experimental tumours.*

The effects of the vaccinia virus (two strains) and of the viruses of fowl plague, herpes simplex, mumps and swine fever (one strain of each) on transplantable tumours (3 mouse tumours and 3 rat tumours) were studied. The experiments were carried out on 900 rats and 750 mice from 1954 to 1959. Incubation period, survival time, and the development, size and morphology of the tumours were observed. The results of grafting in the virusinfected animals and, in a few instances, the effects of some drugs were also studied. In conclusion the authors agree with LEVADITI and NICOLAU, and NICOLAU, that combined application of two or more viruses might be successful in the therapy of cancer.

CAJAL, N., BURDUCEA, O., MATEESCU, S., MARINESCU, GH., CEPLEANU, M. and COPELOVICI, Y. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Development of certain*

experimental viral diseases under the action of radioactive phosphorus (P^{32}) and iodine (J^{131}).

White mice weighing 14–18 g each were inoculated with viral material and 90 minutes later treated, with P^{32} (in Na_2HPO_4) or J^{131} (in NaJ). The individual dose of the radioactive substance was 25 μC administered intraperitoneally. Mortality rate, incubation period, survival time, the pathological lesions and, in the case of influenza virus, the HA titre of the lungs were examined. Aggravation of the experimental disease was observed in the case of the influenza virus, the type 2 poliovirus, the fixed virus of rabies and the herpes simplex virus. Mice under internal irradiation, unlike the control animals, often developed disease when infected with Coxsackie A2 virus intracerebrally, or with type 2 poliovirus subcutaneously or intraperitoneally. Types 1 and 3 of the poliovirus failed to cause disease.

BRONITKI, A., COPELOVICI, Y. and GRUIA, M. (Institute of Inframicrobiology, Academy of Science of the Roumanian People's Republic, Bucharest, Roumania): *The behaviour in the egg and in experimental animals of the influenza virus strains isolated during an epidemic, February, 1959.*

Twenty-nine influenza virus strains were isolated during a wide-spread epidemic in Roumania, in February, 1959. The strains were easily adaptable to the embryonated egg but hardly to the white mouse. Of the strains isolated at the beginning of the epidemic four strains were inhibited by the anti-A serum, seven by the anti-A₂ serum. The rest, mainly those isolated at the peak or later, reacted with both sera in the haemagglutination-inhibition test.

DEREVICI, A., BRONITKY, A., PETRESCU, AL. and SATMARI, C. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *The prophylactic value of the Rumanian anti-influenza vaccines.*

The results of the intranasal vaccination campaigns carried out in the Roumanian People's Republic between 1954 and 1959 are regarded as satisfactory. A vaccine prepared from A1 and B strains was found effective during the influenza A2 pandemic.

DEREVICI, A., BRONITKY, A. and PETRESCU, AL. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *The dynamics of variability of the influenza virus strains isolated in the Roumanian People's Republic, 1954–1959.*

The influenza A, A1 and A2 strains isolated in epidemic and interepidemic periods in Roumania have been characterized. The findings support the view

that the antigenic properties of the epidemic strains are dependent on the particular pre-epidemic immune state of the population.

GRUIA, M., BALMUȘ, G., MITROIU, O. and POPA, M. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Influenza virus inhibiting factors produced by the nasopharyngeal flora.*

The nasopharyngeal microflora of 72 subjects who as influenza contacts had not contracted the disease or had had no respiratory disease during the previous months was cultivated in glucose-blood broth. The first subculture of the bacteria was centrifuged to obtain sterile supernatants, and the supernatants were tested *in vitro* for inhibitory effect against influenza virus. The virus-supernatant mixture was held at + 4° C for 12–14 hours before inoculating into embryonated eggs or white mice. One of the isolates, a Staphylococcus strain, exhibited a very marked inhibitory effect. The bacterium had kept its inhibitory capacity until the 30th subculture; then lost it.

MÎRZA, L., PETRESCU, AL. and ATHANASIU-STROESCU, P. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Influence of anti-influenza vaccination on the unconditioned vascular and respiratory reflexes.*

The circulatory reflexes were investigated by plethysmography, and the respiratory reflexes by pneumography in persons immunized with influenza vaccine. In the first 48 hours following vaccination the plethysmographic curve was inert and vasomotor reactivity reduced or even disappeared. Some troubles in vasomotor reactivity were observed to occur until the end of the 2nd week. No respiratory changes could be demonstrated.

PORTOCALĂ, R., DUMITRESCU, S., IONESCU, N. I., SAMUEL, J. and BOERU, V. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Morphological characteristics of a strain of influenza virus obtained by infection with ribonucleic acid extracted from the homologous virus.*

It had been reported previously that influenza virus was obtained from the allantois sac of embryonated eggs after infection with viral ribonucleic acid prepared from influenza virus according to GIERER and SCHRAMM's technique. Antigenically, the virus so obtained had agreed with the initial strain. In the present work the first and second passage materials were examined by the electron microscope. Apart from minor differences the virus was morphologically identical with the initial strain.

ATHANASIU, P., PETRESCU, AL., GRUIA, M. and SĂRĂȚEANU, D. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Biological changes in a strain of influenza virus during cultivation in mice vaccinated against rabies.*

The initial mouse lung preparation (influenza A/T/53) had an infective titre $10^{-3.1}$. In mice partially immunized against rabies with a fixed virus vaccine, a titre of $10^{-5.7}$ was attained after ten passages. In another experiment, when the initial titre was 10^{-5} , after a single passage in completely immunized mice the infective titre of the lungs was as low as $10^{-3.7}$. The immunogenicity of the lungs was dependent on their infective titre.

NICOLAU, ST. S., CONSTANTINESCU, N., BÎRZU, N. and ZAVATE, O. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania, and Institute of Hygiene, Iassy, Roumania): *Immunity to rabies as estimated by the peritoneal virus neutralization test.*

The neutralization *in vivo* of a massive dose of the Babes strain of fixed virus administered into the peritoneal cavity of experimental animals offers an appropriate test for the demonstration of immunity against rabies. If hamsters, white rats or mice are used as experimental animals, the immunity evoked by vaccination is also demonstrable by the test. In hamsters infected intracerebrally or peripherally with lethal doses of the fixed or street virus the appearance of the clinical symptoms of paralytic rabies are accompanied by the development of a solid immunity of the peritoneum. Unlike the hamster, the mouse, in which animal the disease takes a rapid course, does not develop peritoneal immunity. The peritoneal immunity of the hamster might be regarded as an immune response of the reticuloendothelial system to a transitory viraemia. The *in vivo* rabicidal activity of the peritoneum runs parallel with the *in vitro* neutralizing titre of the blood serum; the brain is about five times less active.

CONSTANTINESCU, N., CAJAL, N., BÎRZU, N., CEPLEANU, M. and ZAVATE, O. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Experimental rabies in the Syrian hamster.*

The Syrian hamster (*Cricetus auratus*) is susceptible to both the street and the fixed virus of rabies. The titres in the hamster's brain (10^{-8} — 10^{-12} at about the onset of the disease) highly exceed the titres usually attained in the rabbit brain. The incubation period is as long as in mice, but the paralytic phase is longer, in certain cases as long as 20 days. The prolonged course of the disease in the unvaccinated hamster allows autosterilization to develop, a phenomenon otherwise observed in partially immune animals only. There are

numerous Negri-bodies to be found in the neurons of hamsters infected with the street virus and giant inclusions are also present, displaying a characteristic internal structure. In hamsters infected with the fixed virus inclusions are demonstrable in Ammon's horn and hippocampus. The use of the hamster in the research and routine laboratory diagnostics of rabies is recommended.

CAJAL, N. and DĂNESCU-POPESCU, G. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *The evolution of experimental rabies and antirabic immunity in animals treated with cortisone.*

Rabbits, hamsters and mice were treated with cortisone during a four-day period when they were inoculated with rabies virus or vaccinated against rabies. The cortisone-treated animals proved to be more sensitive to both the fixed and the street virus than the control ones, as demonstrated by the shorter incubation and surviving periods. The greatest differences were observed in the hamster, the animals in which rabies takes the most prolonged course. The immunity evoked by a vaccine prepared from the Flury strain was definitely enhanced by cortisone; at the time of challenging the cortisone-treated animals had less neutralizing antibody than the control ones.

DEREVICI, A. and PETRESCU, AL. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *The effect of the water-soluble fraction of the bee's "royal gelée" on Ehrlich's ascites tumour.*

The hydrosoluble fraction of the "royal gelée" was mixed with Ehrlich's ascites tumour cells and injected to mice intraperitoneally. In the ascites fluid aspirated at intervals (i) the relative number of tumour cells was low; (ii) the relative number of the tumour cells showing cytoplasmic vacuolization, nuclear pycnosis and/or caryorrhexis was high; (iii) the number of the cells exhibiting mitotic or amitotic division was low; as compared to the ascites fluid from mice inoculated with ascites tumour cells only.

DEREVICI, A. and PETRESCU, AL. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Antiviral activity in the water-soluble fraction of the bee's "royal gelée".*

Two mg of the water-soluble extract of the "royal gelée" given in the allantoic sac immediately after the inoculation of influenza virus completely prevented virus multiplication and did not damage the embryo.

DRĂGĂNESCU, N. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Virological demonstration*

of spring-summer tick borne encephalitis in the Roumanian People's Republic. Study of an isolated strain.

Virus was isolated in white mice from the cerebrospinal fluid of a psychotic patient hospitalized with the symptoms of encephalitis. As demonstrated by neutralization tests carried out with a standard hyperimmune serum, the strain belonged to the group of tick-borne encephalitis viruses. In the mice that had succumbed, encephalitis characterized by perivascular infiltration, neuroglial proliferation and neuronophagia was seen. Mice inoculated intranasally exhibited a pneumoencephalitis characterized by an interstitial infiltration at the level of the lung and by inflammatory lesions in all the neuraxons, specific to this viral disease. After four serial intranasal transfers the virus was still found encephalitogenic in mice when administered by the intracerebral route. Suckling and adult hamsters infected intracerebrally with the virus exhibited the symptoms of encephalitis, particularly paralysis of the hind legs, without tonico-clonic spasms and muscular fibrillations. The pathologic lesions in the brain and meninges were more distinct in the hamster than in the mouse.

SĂRĂȚEANU, D., NASTAC, E., FUHRER, B., OPRESCU, L. and HUNG TAO (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Ornithosis in the Roumanian People's Republic.*

Eight strains of ornithosis virus were isolated; 5 from ducks, 2 from turkeys and one from a goose. The strains were found to be (i) pathogenic for the mouse, particularly by intranasal administration, less pathogenic by the intracerebral or intraperitoneal routes; (ii) apathogenic for the hamster and the rat, even for moderately irradiated rats; (iii) pathogenic for the chick embryo when inoculated either onto the allantoic membrane or into the yolk sac; (iv) the strains agglutinated mouse erythrocytes; (v) fowl antisera prepared against them contained specific neutralizing antibodies, agglutinins and complement fixing antibodies, the latter being demonstrable by the indirect CF test.

In rabbits experimentally infected with the strains intradermal allergy appeared earlier than the CF antibodies did. As demonstrated by serological tests, in Roumania ornithosis is a very common infection among farm workers and persons engaged at poultry packing plants.

MITROIU, O., POPPER, M. and NEGREANU, W. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Differential diagnosis of jaundice accompanying p-amino-salicylic acid (PAS) therapy.*

The serum aldolase test has been found valuable in the differential diagnosis of jaundice appearing during treatment of tuberculosis. In the authors'

opinion, PAS may cause obstructive jaundice and stimulate development of the typical symptoms of infectious hepatitis. The hepatic parenchyma is not affected by PAS.

BALMUS, G., SAMUEL, J., MIRZA, L. and ENACHE, T. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Investigations into a severe Paracoli epizootic affecting young laboratory rats.*

The afflicted animals showed anorrexia, apathy, convulsions and pareses, and succumbed within 48 hours. Congestion in the viscera and necrotic foci in the liver characterized the pathological picture. A strain of Paracoli aerogenes, type intermedium, pathogenic for various laboratory animals was isolated from the heart blood and different organs of the afflicted rats. Since these had been exposed to cold when being transported to the laboratory, authors believe that the cooling diminished the animals' resistance to the otherwise apathogenic bacterium. Passages in the animals with lowered resistance supposedly made the strain virulent for normal animals. The epizootic was stopped by the administration of sulphonamides and tetracycline.

SURDAN, C. and SORODOC, G. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Studies on the virus of the enzootic virus pneumonia of the swine.*

From hogs suffering from enzootic virus pneumonia of swine two strains were transmitted into the yolk sac of embryonated eggs and cultivated there through 12 and 13 serial passages, respectively. The virus killed the embryo in 3 to 9 days. The dead embryos showed haemorrhages, often also necroses, and hepatic hypertrophy and degeneration. Haemorrhages were observed in some yolk membranes, and oedema in different embryonic membranes. Microscopical examination of yolk sac preparations stained according to Herzberg revealed coccoid elementary bodies about $200\text{ m}\mu$ by $250\text{ m}\mu$ in size. In pigs inoculated with the 4th and 11th passage materials, rhinoconjunctival catarrh and fever were observed after an incubation period of from 3 to 11 days; at the same time leucopenia and lymphocytosis developed. Subsequently 80 per cent of the infected pigs developed bronchopneumonia with extensive lymphoid hyperplasia and a tendency to suppuration. Histological lesions were present also in symptomless pigs. The normal pigs isolated as strictly as the infected ones showed no histological lesions.

The virus was reisolated in embryonated eggs from the experimentally infected pigs. Young mice, guinea pigs, hamsters and rabbits proved to be resistant to the virus. The virus obtained either from eggs or from pigs did not agglutinate erythrocytes. Convalescent pigs had neither neutralizing nor complement-fixing antibodies to the virus.

PORTOCALÁ, R., SAMUEL, J. and TÂRNOVEANU, G. (Institute of Inframicrobiology, Academy of Sciences of the Roumanian People's Republic, Bucharest, Roumania): *Demonstration of cytomegalic inclusions in the submaxillary glands of newborns.*

In 4 out of 32 newborns or infants died with various diseases, cytomegalic inclusions were demonstrated in the markedly hypertrophic submaxillary glands. This incidence is similar to that observed in other countries. The demonstrated cytomegalic inclusions indicate the occurrence of cytomegalic inclusion disease in the Roumanian People's Republic.

IVÁNOVICS, G. (Institute of Microbiology, University Medical School, Szeged, Hungary): *Lysogenization of anthrax bacilli by the Anthrax W-phage.*

The *a* mutant of McCLOY's W phage (described as a virulent phage) and the virulent, capsulogenic Vollum strain of *B. anthracis* were studied in order to obtain lysogenic systems. Broth cultures of the bacterium were completely lysed by the phage. Phage titre of the lysate was unusually high, 10^{11} /ml or more. Lysis was followed by secondary growth attaining the maximum bacterium density. Secondary growth was not due to phage-resistant mutants. Moreover, only about 1 or 2 per cent of the secondary population was lysogenic. Resistance of the bacteria can be explained by removal of the phage receptor by the phage. In subcultures, however, in absence of phage the receptor is supposed to regenerate. Such a phenotypic phage resistance has not been known to occur.

All the lysogenic bacteria isolated from the secondary cultures proved to be acapsulogenic. In accordance to this the acapsulogenic R mutant which had been isolated from the virulent Vollum strain was easily lysogenized by the phage. Thus, the mutant of the W phage behaved like a virulent phage if the bacterial cells were capsulogenic and like a temperate phage if the cells were acapsulogenic. Accordingly, the terms "virulent phage" and "temperate phage" may be considered relative. The unstability of the lysogenic complex, *i. e.* the inducibility of the acapsulogenic strain was striking. Induction took place not only in UV-irradiated cultures but also in an atmosphere containing CO₂, on the surface of the agar medium. These observations suggest that the gene determining the capsulogenic characteristic is in near connexion with the prophage. It seems likely that both are located in the same chromosomal segment. Stimuli promoting capsulogenicity may also promote induction.

LANTOS, J., VARGA, I. and IVÁNOVICS, G. (Institute of Microbiology, University Medical School, Szeged, Hungary): *Characterization of some anthrax phages.*

Published in full in this Journal (7, 31, 1960).

VANDRA, E. and GUBA, F. (Diagnostic Laboratory of the "Korányi" Institute for Tuberculosis, Budapest, and Department for Micromorphology of the Research Institute for Technical Physics of the Hungarian Academy of Sciences, Budapest, Hungary): *Examination of some mycobacterium phages by electron microscopy.*

To be published in full in this Journal.

KLEMENT, Z. and LOVREKOVICH, L. (Research Institute for Plant Protection, Budapest, Hungary): *Phage typing of the Pseudomonas species pathogenic for the mulberry tree.*

Published in full in this Journal (7, 113, 1960).

ÁRPAI, J. (Research Institute for Industrial Refrigeration, Bratislava, Czechoslovakia): *Physiological differences among the fractions of pure microbiol cultures.*

Diluted pure cultures of certain bacteria and yeasts, and spores of hyphomycetes, were fractioned by cold sedimentation or slow precipitation. The techniques of sedimentation and isolation are given. Growing resp. germination, fermentation and, particularly, resistance of the cells with different sedimentation rate were examined. Substantial metabolic differences were found between the fractions with the highest and lowest sedimentation velocity. The latter fraction was often less resistant to UV irradiation and formalin than the former one, or the initial cell population. For the induction of mutation in the fractioned cells the existence of an optimum dose was demonstrated which elicited a mutation rate higher than that usually observed for the initial cells.

NAGY, E., ALFÖLDI, L. and IVÁNOVICS, G. (Institute of Microbiology, University Medical School, Szeged, Hungary): *Characterization of megacins.*

Published in full in this Journal (6, 327, 1959).

ALFÖLDI, L., HUSZTI, Zs. and IVÁNOVICS, G. (Institute of Microbiology, University Medical School, Szeged): *Megacinogenesis and nucleic acid synthesis.*

According to SIMINOVICH, DNA synthesis in some lysogenic bacteria induced by UV irradiation stops for a short period, then starts again and goes on vigorously until the lysis begins. In the same period RNA synthesis is constant.

The dynamics of DNA, RNA and protein synthesis were investigated following UV irradiation in the *B. megaterium* strains W 899 (lysogenic); 216 (megacinogenic); 216 M⁻ (amegacinogenic); KM (phage-sensitive, megacin-sensitive) and Mut-C (phage-resistant, megacin-sensitive). The question was whether the supposed genetic relation between megacinogenicity and lysogenic

characteristics would manifest itself in the parallel changes in DNA synthesis following UV irradiation. According to the results DNA synthesis in the megacinogenic bacterium is similar to that demonstrated in irradiated lysogenic bacteria with the only difference that less DNA is synthesised in the former system than in the latter. Following irradiation, however, DNA synthesis in strains KM and Mut-C is very similar to that in the lysogenic bacteria. The transitory inhibition of DNA synthesis after UV irradiation seems to be a general phenomenon, depending neither on the lysogenic nor on the megacinogenic character of the bacterium.

NYIRI, L. and BÖÖR, K. (Biological Research Laboratory of the Hajdúság Pharmaceutical Works, Debrecen, Hungary): *Some problems of the metabolism of B. subtilis.*

The growth of the strain FDA 6633 of *B. subtilis* was investigated in a minimum nutrient medium in the presence of inorganic and different organic nitrogen sources. The microorganism can utilize l-asparagine and pteroyl glutamic acid as the only source of nitrogen only in the presence of both glucose and inorganic phosphate.

L-glutamic acid stimulates the growth of the microorganism, l-aspartic acid and l-asparagine shorten the lag phase and stimulate germination of spores. The amino acids mentioned and ammonia can completely be substituted by folic acid. Caramelized sugar phosphates inhibit the amino acid uptake, protract the lag phase and prolong the growth.

L-glutamic acid is taken up by the FDA strain through the cell wall in the course of an energy consuming process. It is utilized through oxidative deamination. The presence of citrate cycle in the metabolism of the strain has been demonstrated.

MERÉTEY, K. and FÖLDES, J. (Institute of Microbiology, University Medical School, Szeged, Hungary): *Penicillin induced protoplast formation in B. cereus 569 and its mechanism.*

Published in full in this Journal (7, 43, 1960).

SZITA, J. and INCZE, J. (State Institute of Hygiene, Budapest, Hungary): *Examination of the bactericide effect of "Neomagnol" at different pH ranges.*

The influence of pH on the bactericidal effect of "Neomagnol" was studied within a range of pH 3 to 10. Acidified suspensions of both *S. typhi* and *Staphylococcus aureus* were found to exhibit increased sensitivity to the disinfectant. Similar results were obtained in disinfection experiments made by the membrane filtre method. For practical use a 1 to 1000 dilution of "Neomagnol"

acidified to pH 2 by the addition of 30 ml of an 8 per cent solution of HCl/liter is recommended. The free chloride content is not the only factor determining the bactericidal activity of "Neomagnol". Former has namely a maximum at pH 8, latter has not.

GORECZKY, L. (Central Laboratory of the Hungarian State Railway's Hospital, Budapest, Hungary): *Some recent data on the use of preparative electrophoresis.*

Published in full in this Journal (6, 307, 1959).

ZALAY, L. ("Human" Vaccine Production and Research Institute, Budapest, Hungary): *The synergistic and antagonistic effect of antibiotics on bacterium strains used for standard assays.*

In view of the extensive use of the numerous antibiotics and sulphonamides presently available the existence of possible synergistic or antagonistic effects of the commonly applied drugs were examined in experiments *in vitro*. The possible practical relations of the results obtained have been discussed.

KELEMEN, S. and NOVÁK, E. K. (Department of Phthisiology, University Medical School, Budapest, and State Institute of Hygiene, Budapest, Hungary): *Effects of antibiotics on experimental Candida infections.*

In previous experiments the stimulating effect of some antibacterial antibiotics on the growth of *Candida albicans in vitro* was demonstrated. The present study deals with the effect of the same antibiotics on experimental *Candida albicans* infection of mice.

The antibiotic action was found to depend not only on the made and schedule of dosage but also on the particular changes induced by it in both the fungus and the host organism. As revealed by tests *in vitro* stimulated growth was associated with special changes in the enzymatic activities of the antibiotic-treated fungus, while after intraperitoneal infection both speed and way of fungal invasion were modified. The disappearance of intraperitoneally injected India-ink suspension was also found to be promoted in mice treated with antibiotics.

The possible mechanism of the phenomena observed has been outlined.

CSEREY-PECHÁNY, ÉVA, MOLNÁR, B., SOMKUTI, ELISABETH (G. Richter Pharmaceutical Works, Budapest, Hungary): *B₁₂ synthesis of Propionbacteria.*

As concluded by KON and PAWELKIEWICZ (Vienna, 1958), bacteria synthesize vitamin B₁₂ from α -amino-laevulinic acid by way of an unknown intermediary factor which is converted into factor B. Factor B seems to be capable

of being deaminated and reversibly transformed into factor V. The final product, which, depending of the basic substances present, may be vitamin B₁₂ (variable in the nucleotide included) or some complete B₁₂-like factor, is formed from factor B through factor B-ribose-phosphate.

As established by the authors' earlier investigations, the *Propionibacterium* strain isolated in Hungary is able to convert factor V into vitamin B₁₂, and one of the products of both normal fermentation and the fermentation containing factor V, is factor X, *i. e.* the vitamin B analogue with a free carboxyl group. Factor X is, in another approximation, the nucleotide-containing form of factor V.

In order to establish the location of factor X in the biosynthesis, different Co-free deficient factors (factor B, V or X) or one of these factors, or vitamin B₁₂ labelled with Co⁶⁰ were added, and the products were determined by colorimetric paper chromatography or, if a labelled factor was added, by paper chromatography combined with autoradiography, or by electrophoretic analysis.

It has been concluded that (i) factor X arises from factor V, like B₁₂ does from factor B, by combination with the nucleotide; (ii) there exists an equilibrium between factor X and vitamin B₁₂, like that existing between factor B and factor V, based on the reversible amidation of the carboxyl group; (iii) vitamin B₁₂, when added to the fermentation, is converted partly into factor B, partly into factor X, thus, the vitamin is not the final fermentation product. Factor B, factor B-ribose phosphate, vitamin B₁₂, factor X and factor V may be illustrated as a ring where the reversibility of the transformation between the neighbouring products has been proved in almost every phase.



VÁMOS, R. and ZSOLT, J. (Institute of Plant Physiology, University of Szeged, Hungary): *Inhibition of hydrogen sulphide formation in the soil of rice fields.*

In certain kinds of soil inundated for longer periods during the cultivation of rice the prevalence of anaerobic conditions favours reductive processes — especially sulphate reduction — resulting in the formation of H₂S. This substance may cause damage or even total destruction of the rice cultures. Under favourable meteorological conditions with abundant sunshine the plant is able to transport sufficient oxygen to its roots, thus compensating for the toxic effect of the excess H₂S. In the case of unfavourable weather conditions, or in soil in which roots are exposed to the damaging effect of H₂S effective protection of the plant is necessary. For this purpose, as demonstrated by the authors' previous experiments, nitrates are more efficient than quicklime or "red mud". The naturally available or artificially added nitrates, as proved by laboratory tests and field trials, are effective by inhibiting sulphate reduction and oxidizing the H₂S actually formed.

KÖVES, E. and VÁMOS, R. (Institute of Plant Physiology, University of Szeged, Hungary): *Sulphate reduction in the soil.*

Both the facultative (*Proteus, etc.*), and the obligatory anaerobic autotrophic sulphate reducing (*Desulfovibrio desulfuricus*) bacteria were found to be involved in the reduction of sulphates of the soil. The intensity of the sulphate reduction appears to be in close relation to the formation of hydrogen in the soil. To characterize the nature of bacteria and the physicochemical factors probably involved, the fermentation of cellulose by the soil and by the soil bacteria, was examined.

IGALI, S. (Institute of Genetics, Hungarian Academy of Sciences, Budapest, Hungary): *The development and inheritance of chemoresistance in Penicillium chrysogenum.*

The resistance of a genetically homogeneous fungus strain to moderately inhibitory concentrations of certain toxic chemicals was found to be very variable, while treatment with higher concentrations yielded mutant survivors of remarkable resistance being stable even after a longer period of deadaptation. Stable resistance was consequently inheritable in artificially synthesized heterokaryons, in contrast to the variable and moderate tolerance which was transmitted only in the presence of the appropriate inhibitor. In obtaining the resistance of this type both the phenotypic (cytoplasmic) and genotypic (nuclear) mechanisms seem to take part.

HORVÁTH, I., GADÓ, I. and SZENTIRMAI, A. (Research Institute of the Pharmaceutical Industry, Budapest, Hungary): *Isoleucine synthesis by Streptomyces rimosus.*

Str. rimosus, when growing in media containing threonine, accumulates isoleucine and this accumulation can be enhanced by the addition of alanine. The first step of isoleucine synthesis is probably the deamination of threonine into β -keto-butyric acid. The deaminase, a constitutive enzyme, catalysing this step is present in the cell-free extract. A method for purification of the enzyme has been described and the enzyme has been characterized. Investigations are in progress to demonstrate further enzymes involved in the synthesis of isoleucine.

INGRAM, M. (Low Temperature Research Station, Cambridge, England): *Studies on benzoate-resistant yeasts.*

Published in full in this Journal (7, 95, 1960).

PELC, A. and SZÉP, I. (Research Institute for Fermentation Industry, Budapest, Hungary): *Relation of the respiration of yeast cells to the concentration of dissolved oxygen.*

The effect of motion on the concentration of dissolved oxygen in the medium was investigated partly in the Warburg apparatus, partly by TÖDT's method. Changing the oxygen concentration between 1 and 28 mg had no influence on the yeast cell respiration within 1 hr. Cultivation for eight hours in media saturated with oxygen shifted metabolism towards respiration. Solution of oxygen gas in the medium and diffusion of the dissolved oxygen into the cell was more effectively promoted by stirring than by raising the concentration of oxygen. Thus the intensity of stirring is a more important factor in the respiration rate of the yeast cell than increasing the medium's surface.

VÁMOS, E. (Research Institute for the Fermentation Industry, Budapest, Hungary): *Role of oligosaccharides in the anaerobic metabolism of yeasts.*

The role of the oligosaccharides arising during the acid hydrolysis of starch in the aerobic metabolism of yeasts depends on species and variety. BAKER's yeast utilizes only mono- and disaccharides, *Saccharomyces Saké Yabé* also trisaccharides, while *Schizosaccharomyces pombei* utilizes even large molecule oligosaccharides. During fermentation with *S. pombei* in a medium containing starch hydrolysate, two oligosaccharides not present in the initial medium were demonstrated.

The mechanism of the production of the two oligosaccharides have been discussed, pointing out that fermentation of the di-, tri- and tetrasaccharides of the medium does not take place directly but by way of an intermediate resynthesis.

FÁBRI, ILONA (Institute for Canning and Refrigeration Research, Budapest, Hungary): *Metabolism of osmophilic yeasts in concentrated fruit juices.*

The carbohydrate assimilation of 22 single-cell cultures was examined by turbidimetry. Glucose was utilized by 21, saccharose by 16 and galactose by 3 of the strains under study. The rate of fermentation and inversion of saccharose as determined by turbidimetry was confirmed by paper chromatography. Fermentation by the osmophilic strains was moderate and incomplete in 30 per cent saccharose solutions. In non-agitated cultures formation of considerable amounts of polyhightoxyalcohol was observed, at a sugar concentration of 10 per cent. None of the strains produced glycerol in 30 per cent sugar solution. The pectine decomposing activity of the strains was weak.

MINÁRIK, E., LAHÓ, L. and NAVARA, A. (Institute for Ampelological Research, Bratislava, Czechoslovakia): *Determination of the sugar assimilating activity of wine-yeasts.*

Though carbohydrate metabolism is one of the most important biological characteristics of microorganisms for its examination no sufficiently reliable and reproducible methods are available. A new, simple and reliable method making use of paper chromatography is recommended and its results are presented. The method suits itself also for verification of the results of fermentation tests.

VAS, K. and FARKAS, J. (Institute for Canning and Refrigeration Research, Budapest, Hungary): *Some direct effects of ionizing radiations on the yeast cell.*

The direct changes in the yeast cell caused by large doses of X-rays and electronic radiation have been studied by paper chromatography and colorimetry of the free reserve amino acids released from the cell and by measuring the dehydrogenase activity of *Sacch. cerevisiae* suspensions with triphenyl tetrazolium chloride. Leakage of amino acids was found to become perceptible from about 280 krad. Increasing the dose resulted in a marked increase in the diffusion of amino acids up to about 1100 krad. At equal doses, X-rays were more effective than electron beams. Dehydrogenase activity of yeast suspensions showed a considerable decrease with increasing doses up to around 140—210 krad. Further increase in the dose, up to about 1100 to 2200 krad, strongly enhanced the formation of triphenyl formazan; above this level, especially around 4500 krad, a decrease was again noticed. The phenomena observed have been explained by disturbances in the enzyme systems of the dying yeast cell, an increase in permeability, and, at the highest dose levels, by the inactivation of enzymes.

JENEY, E., SZENDREY, S. and VIC, E. (Institute of Hygiene, University Medical School, Debrecen, Hungary): *Nucleic acid content of *Saccharomyces cerevisiae* after treatment with carbon monoxide, potassium cyanide and sodium azide.*

In previous experiments changes in some biological functions of *Saccharomyces cerevisiae* were examined in the presence of carbon monoxide, potassium cyanide or sodium azide. Examinations were performed in Einhorn-tubes, van Ittersen-Cluyver manometer, Warburg apparatus and microcalorimeter. In the present experiments made under similar conditions the changes in the nucleic acids of yeast cells were studied by a method combined of SCHMIDT-TANNHÄUSER's and SCHNEIDER's procedure.

It has been found that sodium azide induced but insignificant changes in the deoxyribonucleic acid content, while potassium cyanide and carbon monoxide caused a tenfold resp. 17 to 18 fold increase in the same component. The

ribonucleic acid content decreased by 60 per cent in the presence of sodium azide, increased by 17 per cent in the presence of carbon monoxide, and no change was produced by potassium cyanide. The findings were supported by experiments making use of isotope-labelled compounds.

PAZONYI, B., BÁLINT, E., SZÉP, IVÁNNÉ, MÁRKUS, L. (Institute of Genetics, Hungarian Academy of Sciences, Budapest, and Research Institute for the Fermentation Industry, Budapest, Hungary): *Biological standardization of baker's yeast.*

In order to elaborate an appropriate method for the qualification of yeast strains the properties of *Saccharomyces cerevisiae* strain M. XII. used in the production of baker's yeast, have been studied.

The yeast used in the experiments was precultivated under strictly defined conditions. Respiration-fermentation rate was estimated by the Warburg method, fermentation rate by Iterson-Kluyver's fermentometer, germination rate by cell counts, sugar assimilation, qualitative amino acid and nucleic acid requirement by photometry. The method which includes the detailed examination of the biological properties of yeast has been found suitable for the qualification of yeast strains, as supported also by model cultivation experiments. The results of the latter were in full agreement with those of the biological method which can be used also for the examination of the adequacy of other cultivation conditions, e. g. the medium's composition.

For the qualification for industrial use of a baker's yeast strain it is not necessary to perform all the tests listed above. It suffices to establish the respiration-fermentation, germination and multiplication rates.

SZÉP, IVÁNNÉ (Research Institute for the Fermentation Industry, Budapest, Hungary): *Adaptation of yeasts to disinfectants.*

Two strains of *Saccharomyces cerevisiae* used for distillation were adapted through a period of 12 months to formaldehyde, chlorine and cetyl pyridinium bromide, respectively. By gradual increase of the disinfectant's concentration resistance was made to develop against as high concentrations as those inhibiting the multiplication of the acid forming bacteria which often cause contamination in the industry.

The adaptation was successful as demonstrated in fermentation experiments. The adapted strains proved to be capable of growing in media containing 0.045 per cent formalin, or 0.06 per cent active chlorine or 0.01 per cent cetyl pyridinium bromide. In the same media the initial strains did not ferment. In the media containing disinfectant the lag phase of the adapted strains was prolonged, but in the logarithmic phase fermentation was as intensive as in the case of the unadapted strains growing in media containing no disinfectants.

ÁSVÁNY, Á. and ZSOLT, J. (Institute for Ampelological Research, Budapest, and Institute of Plant Physiology, University of Szeged, Hungary): *Genetical investigation of wine yeasts.*

The length and width of the cells was measured in one-cell and one-spore cultures of a Hungarian wine yeast ("Tokaj 15"). A culture medium malt extract with a density of 10 Bllg was applied. Measurements were undertaken on the first, third, fifth and tenth days. Cultures grown at different temperatures (16°, 25°, and 33° C) were also investigated.

Statistical evaluation of the findings showed that the form of the cells is rather variable even in one-spore cultures. This makes it difficult to obtain identical data at different times. As a rule, with an increase of the length the width diminished and length was more variable than width.

ZSOLT, J. (Institute of Plant Physiology, University of Szeged, Hungary): *Critical examination of the use in yeast taxonomy of some metabolic characteristics.*

Experiments on the alcohol and nitrate assimilation and the "starch" synthesis of yeasts are presented. Differences in the ethanol assimilation observed by different authors are to be attributed to methodical differences. A positive ethanol assimilation test as defined by LODDER and KREGE VAN RIJ means utilization of ethanol as the sole source of carbon, while according to KUDRIAVZEV it means utilization of the carbon of ethanol, thus a more abundant growth if ethanol is added to a medium which by itself is able to support a definite but weak growth.

Observation of the nitrate assimilation is not always possible, because binding of the atmospheric nitrogen — observed also by the author — disturbs the usual tests. Synthesis of "starch" is sometimes uncertainly observable; its taxonomic significance seems questionable.

VÖRÖS-FELKAI, G. and NOVÁK, E. K. (State Institute of Hygiene, Budapest, Hungary): *The occurrence of yeasts in human clinical specimens.*

A survey is given on fungi isolated from clinical specimens from patients with suspected mycosis or mycosis associated with different diseases. The incidence of different fungi exhibited a remarkable increase, chiefly of those which earlier had seldom been observed. Three of the isolated species, namely: *Torulopsis groppengiesseri*, *Hansenula subpelliculosa*, *Rhodotorula flava* had not been described to occur in specimens of human origin.

JÁRAI, M. ("Chinoin" Pharmaceutical and Chemical Works, Budapest, Hungary): *Genetic recombination in Streptomyces aureofaciens.*

Eleven biochemical mutants with requirements for arginine, methionine, cysteine and amino N have been produced. Streptomycin resistance, pigment

production, conidia colour and the ability to produce antibiotics were determined for each mutant.

In addition to the antibiotic production of the prototrophic recombinants from different biochemical mutant-pairs, the respiration and metabolism of these were also examined.

In 11 per cent of the prototrophic recombinants antibiotic production was remarkably increased. The increase was 20 fold as compared to the parental auxotrophs and 40 to 50 per cent as compared to the starting prototrophic industrial strains.

The increase in antibiotic activity was especially marked in the case of recombinants originating from a definite biochemical mutant pair. Thirty-two per cent of the recombinants with high activity were derived from the N-4 methionine deficient and N-9 arginine deficient complementary auxotrophs. Of the recombinants from these mutants 41 per cent elicited a high antibiotic production.

Recombinants of high activity were produced mainly by mutants obtained in different way from prototrophic strains different in origin, e. g. by means of UV irradiation the one, and X irradiation the other.

NYIRI, L. (Biological Research Laboratory of the Hajdúság Pharmaceutical Works, Debrecen, Hungary): *Morphological changes of Streptomyces rimosus strains in deep cultures.*

The morphological changes of *Str. rimosus* RS-21 sporogenic and *Str. rimosus* LSzT 118 non-sporogenic variants were studied in aerated deep cultures. In the first generation of the strain RS-21 9 morphological phases were differentiated. The granule formation characteristic of Streptomyces could not be observed in the deep cultures of the strain LSzT 118, neither in the second deep culture generation of the RS-21 strain.

Thus two ways of change of generations may be observed, (i) spore — long hyphae — granule — short hyphae — spores; (ii) Spore — long hyphae — short hyphae — spores.

Sporulation took place in both strains with the fragmentation of the hypha. The morphology of development of *Str. rimosus* was very similar to that described by CARVAJAL for *Str. griseus*. The scheme suggested by KLIENE-BERGER-NOBEL for the morphological changes of Actinomycetes was not valid for the strains of *Str. rimosus*.

PAZONYI, B., CHOLNOKY, L., BÁLINT, E. K. and SZÖLLÖSY, I. (Institute of Genetics of the Hungarian Academy of Sciences, and Institute of Chemistry, University Medical School, Pécs, Hungary): *The carotinoids of Rhodotorula glutinis (Fres.) Harrison and of its mutants.*

Some carotinid mutants of *Rhodotorula glutinis* viz. purple (*purpurea*), arange (*aurantiaca*), miniate (*miniata*), Spanish orange (*aurantiaca hispanica*), salmon-colour (*salmonicolor*), saffron-coloured (*grocea*), yellow (*lutea*), oriental rose (*rosea orientis*) and ivory (*eburnea*) were obtained by UV irradiation. Each of the mutants was cultivated in an identical manner, and the carotinid components were determined in qualitative and quantitative tests. Some carotinids hitherto not demonstrated in *Rhodotorula glutinis* were found to occur both in the initial culture and in the mutants.

NOVÁK, E. K. (State Institute of Hygiene, Budapest, Hungary): *The dehydrogenase activity of malonate-cultivated Candida albicans.*

According to earlier experiments, numerous species of fungi including *Candida albicans* utilize malonic acid as the only source of carbon. In the present experiments the dehydrogenase activity on different substrates of malonate-cultivated cells was determined.

The malonate-cultivated cells had a high endogenous substrate-dehydrogenase activity which was less sensitive to malonic acid than the activity of the glucose-cultivated cells. The activity measured in the presence of succinic acid was weaker than the endogenic one, and that measured in the presence of both malonic and succinic acids was hardly demonstrable. Activity measured on diethyl malonate exceeded the endogenic value. The activity on diethyl malonate, acetic acid, ethanol, citric acid, fumaric acid, lactic acid and oxalic acid were inhibited by both succinic acid and malonic acid.

NOVÁK, E. K. (State Institute of Hygiene, Budapest Hungary): *Changes induced by antibacterial and antimycotic substances in the fermentative activity of Candida albicans.*

The effect of some antibacterial and antimycotic substances on the endogenous substrate dehydrogenase activity of *Candida albicans* is sometimes the opposite of their effect on the growth of the same organism. The influence of such substances on the glucose and maltose fermentation by *Candida albicans* has been studied. Some of the substances proved indifferent; others stimulated or inhibited the fermentation of both sugars. Several substances influenced glucose and maltose fermentation differently. It has been concluded that a substance though inhibiting the growth of an organism may stimulate its sugar fermentation, and vice versa.

KAROLČEK, J., ODLER, I. and DRACOVICOVÁ, M. (Institute of Epidemiology and Microbiology, Bratislava, Czechoslovakia): *The immune response of humans and animals to typhoid vaccination and natural infection, with particular regard to the bactericidal activity of the serum.*

The dynamics and mutual relation of immune levels in humans and rabbits as determined by different tests before and after vaccination with different typhoid vaccines has been discussed. Specific agglutinin titres (O, Vi, H), specific opsonino-phagocytic index (FI), mouse protection, and bactericidal activity *in vitro* of the serum were titrated. The results were compared with the immune responses of typhoid carriers and rabbits immunized with typhoid bacteria sensitized with Besredka's method as well as with the immunological status of typhoid patients and of persons who had typhoid fever.

In the immunized persons no discrepancy in the course of the different immune responses was observed whereas the response of rabbits immunized with typhoid vaccines or with sensitized typhoid bacteria, and the response of typhoid carriers was opposite in sense of bactericidal activity on the one hand and of specific antibody titres and FI on the other, *i. e.* bactericidal activity showed a substantial decrease while antibody titres and FI were rising.

Possible causes and significance of this discrepancy as well as the role of the bactericidal activity of serum in the typhoid immunity has been discussed. The question has been raised how the above results can be used in a reliable potency test for typhoid vaccine.

RAUSS, K. and KÉTYI, I. (Institute of Microbiology, University Medical School, Pécs, Hungary): *Certain antagonistic effects of the E. coli flora.*

A foreign *E. coli* strain administered orally cannot be implanted into the bowel of the mouse, except if its own intestinal *E. coli* flora has been eliminated by antibiotic (streptomycin) treatment. All the *E. coli* strains tested by the authors proved antagonistic, even those of the same serotype, regardless whether the *E. coli* flora was normal or artificial.

The antagonism was not related to immunity or colicin production, and was considered an interference phenomenon. In mice infected simultaneously with two strains of *E. coli*, one of the strains became predominant while excretion of the other was temporary.

In preliminary experiments the artificial *E. coli* flora prevented implantation of *Klebsiella* and *Shigella* whereas *Klebsiella* and *Shigella* were not antagonistic to the *Escherichia* genus. No antagonism was observed among the genera *Escherichia*, *Proteus* and *Salmonella*.

The significance of the antagonism in the human *E. coli* flora, the possibility of a mutaflore therapy and some questions of double infection have been discussed.

RAUSS, K. and VÖRÖS, S. (Institute of Microbiology, University Medical School, Pécs, Hungary): *Production of hydrogen sulfide by intestinal bacteria.*

The contradictory data on the H_2S production by *Proteus morganii* made it necessary to study which component of peptone was responsible for

this important biochemical reaction. *Proteus morganii* was found to grow well and produce H_2S on synthetic media containing either cystine or cysteine, growth factors, glucose and inorganic substances. Methionine supported growth but did not induce H_2S production. Most of the so-called H_2S -negative bacteria behaved similarly. Peptone preparations excellently suitable to demonstrate H_2S production were found to contain methionine but neither cysteine nor cystine. They contained, however, sulphate as an inorganic source of sulphur. Peptone preparations made sulphate-free by adding $BaCl_2$ did not support H_2S production. In this function sulphate could be replaced by sodium thiosulphate.

It has been concluded that the so-called H_2S -negative group of Enterobacteriaceae is capable of producing H_2S from cysteine and cystine but not from inorganic substances. However, cysteine and cystine are lacking in the usual peptone preparations. H_2S -positive bacteria, on the other hand, are able to produce H_2S from inorganic substances. In the usual peptone preparations they utilize sulphates as the source of sulphur.

ANGYAL, T. (Institute of Microbiology, University Medical School, Pécs, Hungary): *Characterization of some Staphylococcus strains isolated from infantile enteritis.*

Three hundred Micrococcus strains which had been isolated from faecal specimens of children admitted with symptoms of enteritis to the Paediatric Department, were investigated. Ninety-eight per cent of the strains belonged to the group *Staphylococcus aureus* or *albus* (BERGEY 1957) and appeared to be pathogenic according to the coagulase, hyaluronidase, and phosphatase tests. The strains were serologically different. The high incidence of type "b", the relatively scarce occurrence of the "e" antigen strains and the great number of inagglutinable strains as compared with strains from other sources, were remarkable. The strains were highly resistant to streptomycin, chloramphenicol, aureomycin and terramycin, while only one strain was resistant to neomycin.

ZOLTAI, N., JANKÓ, M., BALLÓ, T. and LÓRÁNT, O. (State Institute of Hygiene and Árpád Hospital, Budapest, Hungary): *On the pathological role of Entamoeba histolytica strains in Hungary.*

Most of the authors regard the *E. histolytica* strains of the temperate zone as apathogenic. Nevertheless, because of some clinical cases in Hungary and the about 20 per cent incidence of carriers among children at country schools, the pathogenicity of *E. histolytica* strains occurring in Hungary was investigated. During a 3-year period of survey a 20 per cent incidence of *E. histolytica* infection was demonstrated among patients with the clinical diagnosis of dysentery. One third of these cases was negative for Shigella. Thirty-two per cent

of the cases yielding only *E. histolytica* were severe in contrast to the 3 per cent severe cases among those infected only with bacteria. The gravest cases were those infected by both *E. histolytica* and Shigella. Among the inhabitants of the area where the patients with dysentery came from, a 4 per cent incidence of *E. histolytica*, i.e. one fifth of the frequency among the patients, was detected.

Of the patients with chronic colitis 33 per cent was found infect by *E. histolytica*. Specific treatment yielded excellent results even in cases with a history of several years.

Using strains isolated from patients with chronic colitis, successful infection of young cats was achieved (see p. 107).

As to the clinical diagnosis of amoebiasis under the temperate zone, the cooperation of physicians and parasitologists was fruitful, as the symptoms were often atypical and amoebiasis was often associated with infections by other enteric parasites.

On the grounds of statistical analyses the *E. histolytica* strains which occur in Hungary are regarded facultatively pathogenic, though in this instance pathogenicity seems to be much more a result of a particular situation of the hostorganism than a *sui generis* characteristic. A similar situation is probably prevailing in other countries in the temperate zone.

BAKÁCS, T. and JANKÓ, M. (State Institute of Hygiene, Budapest, Hungary): *On the pathological role of Entamoeba histolytica strains in Hungary. II. Animal experiments.*

Published in full in this Journal (7, 107, 1960).

UJHELYI, K. (State Institute of Hygiene, Budapest, Hungary): *Preparation and examination of the histolytic action of the proteolytic enzyme of V. cholerae.*

Some strains of *V. cholerae*, particularly those of high virulence are greatly active in producing an enzyme-liquefying gelatine. The enzyme was purified and concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ and fractional precipitation with alcohol. The purified enzyme was stored without any significant loss in activity. The sera of rabbits immunized with the purified preparation were able to suspend even at high dilutions the characteristic effect of the enzyme, viz. destroying the interstitium of the small intestine. This capacity of the enzyme inhibitor was clearly demonstrable in unstained histological sections. Dilute solutions of the enzyme preparation could be used in preparing dispersed cell cultures from the guinea pig's small intestine. Preparation of a dispersed kidney cell culture needed more concentrate enzyme.

It is suggested that the proteolytic enzyme is an important factor in the pathomechanism of Asiatic cholera as well as in the immunogenicity of *V. cholerae*.

SERÉNY, B. (State Institute of Hygiene, Budapest, Hungary): *S. sonnei colonies with radial pattern.*

Published in full in this Journal (6, 179, 1959).

RÉDEY, B. and CSIZMAZIA, F. (Public Health and Epidemiological Station, Veszprém, Hungary): *Attempts to demonstrate pathogenic intestinal bacteria by the guinea pig eye test.*

Published in full in this Journal (7, 11, 1960).

WEISSFEILER, J. (State Institute of Hygiene, Budapest, Hungary): *Studies on a new strain for tuberculosis vaccine production.*

Published in full in this Journal (7, 77, 1960).

ERDŐS, L. (State Institute of Hygiene, Budapest, Hungary): *The possibility of inducing immunity in 3—4 months old infants by diphtheria-tetanus-pertussis adsorbed vaccine.*

In Hungary vaccination of infants with the diphtheria-pertussis-tetanus adsorbed vaccine is desirable at 3 months instead of between the 6th and 11th months of age since about 80 per cent of the fatal cases of whooping cough occur among infants under one year of age. Two weeks following the basal immunization consisting of two inoculations at an interval of two weeks the authors found no difference in the antibody titre of infants vaccinated at 3 month of age and those vaccinated between the 6th and 11th months of age. (The same preparation was used throughout.) Eight months later, however, the titres particularly those to diphtheria and pertussis, were found to be lower in the younger age group than in the older one. Thus revaccination at 12 months is recommended.

The immune response to revaccination in the younger group was as good as in those who had received basal immunization during the second half year of their life. A more effective prevention of whooping cough may be expected from giving children a basal immunization consisting of two inoculations at 3 and 4 months of age, respectively, and a booster dose at 12 months of age.

PUSZTAI, Zs., JOÓ, I. and KISS, I. ("Humán" Vaccine Production Research Institute, Budapest, Hungary): *The influence of ion-exchange resins on the growth of Bordetella pertussis.*

The effects of anion exchange resins in the hydroxyle, chloride or phosphate cycles (Amberlite IRA 400 and Amberlite IRB) on the growth of some strains of *B. pertussis* in shaken, fluid media were investigated. The resins,

regardless of cycle, stimulated the growth of the bacterium with no influence on the phase I characteristics. The minimum inoculum sufficient to initiate bacterial growth was found substantially less in media containing resin than in the control media. The mechanism of the resins' effect was also investigated.

LOVAS, B. (Department for Micromorphology of the Research Institute for Technical Physics of the Hungarian Academy of Sciences, Budapest, Hungary): *Changes in the morphology of Salmonella typhi-suis var. Voldagsen in a highly viscous culture medium.*

It was demonstrated by electron microscopy that bacterial cells passing through a 2 per cent or more concentrated gel of 4–8 mm thickness showed an unusual increase in the number and size of flagellae. The huge bundles of flagellae were situated almost exclusively near the poles. Passing columns of gel thicker than 8 mm the bacteria lost the capacity of dividing, granulation appeared in the cells, swelling and disintegration of the cell and finally, disappearance of the cell wall were observed. Similar changes were not observed in cells which had been kept immotile.

LOVAS, B. (Department for Micromorphology of the Research Institute for Technical Physics of the Hungarian Academy of Sciences, Budapest, Hungary): *Optical and electron microscopical morphology of Salmonella typhi-suis var. Voldagsen treated with a sublethal doses of penicillin.*

Coverglass cultures of *Salmonella typhi-suis* var. Voldagsen were damaged by sublethal doses of penicillin. Bacterial growth and the morphological changes were observed under the optical microscope with the stage heated. Characteristic protoplast-like forms were seen. If the cultures had been contaminated with a penicillinase-producing strain of *B. cereus*, these forms disappeared and the normal Voldagsen bacteria began to grow intensely. The above phenomena were re-examined by phase contrast and electron microscopy.

NIKODÉMUSZ, I. and CSABA, K. (Institute of Nutrition, Budapest, Hungary): *A case of botulism verified by laboratory tests.*

The demonstration of *Cl. botulinum* and its toxin was attempted in a brawn the consumption of which had caused a typical case of botulism and several cases of mild disease. The filtered suspension of the brawn killed the mice inoculated with it; this lethal effect could be prevented with *Cl. botulinum* antitoxin. The brawn contained about 1800 mouse LD₅₀/g. Although toxin was demonstrated in the filtrate of the culture made from the brawn, *Cl. botulinum* could not be isolated because of the presence of different aerobic and anaerobic bacteria.

SYMPOSIUM ON RESISTANCE TO ANTIBIOTICS ORGANIZED BY THE HUNGARIAN ACADEMY OF SCIENCES

September 25, 1959, Budapest

Abstracts of Papers Presented

JENEY, E. (Institute of Hygiene, University Medical School, Debrecen, Hungary): *Theoretical problems of resistance to antibiotics.*

PETRÁNYI, GY. (Department of Medicine, University Medical School, Debrecen, Hungary): *Clinical problems of resistance to antibiotics.*

YERMOLYEVA, Z. V. (Central Institute for Antibiotic Research, USSR Ministry of Health, Moscow, USSR): *Resistance to Antibiotics.*

Based on her own experiments and the experience of other in the USSR the author recommends the following measures to make the sanitary and prophylactic use of antibiotics more efficient.

(i) Introduction of more effective preparations and combined antibiotic therapy in order to prevent the development of resistance.

(ii) Studies on the role of resistant and dependent forms as well as of L-forms of bacteria in the aetiology epidemiology, and pathogenesis of different diseases.

(iii) Resistant Staphylococci isolated at hospitals are to be tested for phage type, virulence, and penicillinase activity, and characterized biochemically. Staphylococcus toxoid should be applied to prevent mastitis. Complex preparations should be used in the treatment of neonatal diseases and carriers. In maternity homes and surgical wards the strictest sanitary and hygienic measure should be observed and continuous bacteriological control should be introduced.

(iv) Resistance to antibiotics should be determined by the most sensitive methods (bacteria are to be tested with three concentrations of each antibiotic, and several penicillin preparations should be used).

(v) The method of phagocytosis is recommended to study the antibiotic capacity of human and animal tissues and cells.

(vi) New antibiotics and new preparations of biomycin, streptomycin and penicillin are necessary to overcome the resistant forms of bacteria.

(vii) Stock of antibiotics is to be brought about including antibiotics effective against the penicillin-resistant forms of microorganisms (oleandomycin is such an antibiotic).

(viii) The antibiotics for local use and those for use by other routes should be different. The periodical change of antibiotics in common use is also recommended.

HABÁN, GY. (State Institute of Hygiene, Budapest, Hungary): *The incidence of antibiotic-resistant bacteria in Hungary.*

The sensitivity to antibiotics of 12 176 strains isolated from 10 275 clinical samples during the period from January 1, 1957, to June 30, 1959, at the Department of Bacteriology of the State Institute of Hygiene was determined.

The average penicillin resistance of Staphylococci decreased from 65 to 61 per cent during the last two and a half years. Resistance was found to depend on the source of the organism; Staphylococci from punctates were resistant to penicillin in 69 per cent, while those from the ear and the nasopharynx in 57 and 44 per cent, respectively. As the latter samples were taken mostly from healthy persons, the existence of a correlation between pathogenicity and penicillin-resistance of Staphylococci seemed to be very probable. Resistance to streptomycin, chloramphenicol and tetracycline exhibited some slight increase as compared to the previous data.

The antibiotic-resistance of *E. coli dyspepsiae* was also studied. The resistance to chloramphenicol rose to 95 per cent for *E. coli B 4* and 53 per cent for the other *E. coli dyspepsiae* strains. An increased resistance to terramycin, aureomycin, erythromycin and tetracycline was observed for both the *E. coli B 4* and the other *E. coli dyspepsiae* strains. On the other hand only 2 per cent of the *E. coli B 4* strains isolated were resistant to neomycin and polymyxin B. In certain cases resistance to the antibiotics recently applied appears to have developed in a relatively short period. The phenomenon might probably be explained by the use of antibiotics on a much wider scale than earlier.

BARNES, E. M. (Low Temperature Research Station, Cambridge, England): *Resistant streptococci in the gut of chicken fed chlortetracycline.*

The faecal streptococci of normal chicken are mostly *Str. faecium*, with some *Str. faecalis* var. *liquefaciens*; all are sensitive to tetracyclines. In birds fed chlortetracycline, a non-liquefying strain of *Str. faecalis* was predominant. This however belonged to the same serological strain as the liquefying variants in control birds.

KURYLOWICZ, W. (Institute of Antibiotics, Warsaw, Poland): *The natural resistance against antibiotics as an aid to the differentiation of attenuated Mycobacteria.*

To differentiate the group of attenuated tubercle bacilli, mainly BCG substrains of different origin, their resistance to antibiotics and selected tuberculostatics was determined.

The experiments were performed on three groups of acid fast *Mycobacteria*: (a) vigorously growing, nonpathogenic strains (chromogenic and non-chromogenic); (b) slowly growing, pathogenic tubercle bacilli of human and bovine type; (c) slowly growing, attenuated tubercle bacilli of bovine type — BCG substrains, obtained from various laboratories.

The strains were differentiated on the grounds of (i) natural resistance to antibiotics and to some known synthetic tuberculostatic compounds; (ii) morphology of macro- and microcolonies on egg and well-defined media; (iii) metabolism of C^{14} labelled sodium acetate and glucose, (iv) the amount of lipid-poly saccharide fraction (pmko); (v) immunological behaviour in animals, by means of virulent *M. tuberculosis* H37Rv and Vallée labelled by C^{14} ; (vi) susceptibility to a specific mycophage (D-29).

The antibiotics bacitracin, polymyxin B, chloromycetin, chloro- and oxy-tetracycline, dihydrostreptomycin, kanamycin, cycloserin, homomycin, angust-mycin and penicillin were used. From the synthetic compounds NIH and PASA were examined.

The three above mentioned groups of *Mycobacteria* were easy to differentiate by means of tuberculostatic agents. Group (a) was susceptible to antituberculous antibiotics, but resistant even to high concentrations of PASA and INH. Group (b) and (c) were susceptible to both kinds of antibiotic compounds. In group (c), which had been considered as rather uniform biologically, the following differences were found among the BCG substrains belonging to this group:

(i) in the susceptibility to antibiotics; (ii) in the uptake of C^{14} from well-defined media containing radioactive sodium acetate and glucose; (iii) in immunochemical composition and immunological behaviour; and, (iv) in the susceptibility to the specific mycophage action.

Between the above mentioned characteristics of attenuated *Mycobacteria* few correlations have been established.

The natural resistance of attenuated *Mycobacteria* has been established.

The natural resistance of attenuated *Mycobacteria* to tuberculostatic compounds has some differential value for the classification of this group of microorganisms.

OPEL, H. (Institute for Phytopathology, German Academy of Agricultural Sciences, Aschersleben, Germany): *Enhanced growth of microorganisms on the borders of inhibition zones.*

When testing an antibiotic by the agar-diffusion method, enhanced growth may often be observed on the margin of the inhibition zone. This

phenomenon has been described by several authors and different explanations have been suggested. As an acceptable explanation the stimulating effect of subliminal antibiotic concentrations was regarded.

The analysis of the growth factors of *B. subtilis* used as test organism revealed that the so-called marginal growth stimulating effect — which is entirely independent of the antibiotic action — may easily be regarded a direct effect of the antibiotic tested. A marginal stimulating effect can be observed also along growth margins artificially made on the inoculated agar surface. For the latter purpose the following two methods may be employed, (i) covering a part of the agar surface by small pieces of coverglass; or (ii) cautiously replacing a small area of the inoculated agar plate by fresh agar. The marginal growth stimulation produced by either of these methods may be the result of the greater amount of nutrients available (Effect No. 1), or else that of the rapid decrease of autotoxic metabolite concentration by diffusion (Effect No. 2). The latter was proved for *B. subtilis* by demonstrating a 52 per cent growth stimulation photometrically. Patulin caused 22 per cent stimulation with the same test organism. An exact quantitative analysis is not always possible.

JENEY, E., GÉDER, L. and SZABÓ, M. (Institute of Hygiene, University Medical School, Debrecen, Hungary): *Phage types of antibiotic-resistant pathogenic staphylococci isolated from the physicians and patients of the Debrecen University Hospitals.*

Four hundred and seven *Staphylococcus* strains were isolated from the throat, faeces and fingers of physicians, nurses and other members of the staff of the University Medical School of Debrecen and strains 278 from clinical samples from patients of the same institutions.

The strains were examined for coagulase production, mannite fermentation, haemolytic action and gelatine liquefaction. Their sensitivity to penicillin, streptomycin, aureomycin, terramycin, chloramphenicol, erythromycin and sulphonamides was determined. The strains were phage-typed using 21 standard type phages. Most of the pathogenic strains belonged to phage type I, viz. 23.2 per cent among the staff and 35.3 per cent among the patients, a total of 58.5 per cent. Type IV strains were unfrequent.

Most of the strains resistant to penicillin belonged to types I, II and III, while most of those resistant to streptomycin, chloromycetin, terramycin, aureomycin or other antibiotics tested belonged to type III.

VÁCZI, L., JENEY, E. and GÉDER, L. (Institute of Microbiology, University Medical School, Debrecen, Hungary): *Possibilities of phage-typing of polyresistant Staphylococcus pyogenes aureus strains.*

Published in full in this Journal (6, 249, 1959).

VÁCZI, L., HADHÁZI, GY., KATONA, M. and FODOR, M. (Institute of Microbiology, University Medical School, Debrecen, Hungary): *Surface characteristics and resistance to antibiotics in Staphylococcus pyogenes aureus strains.*

Published in full in this Journal (6, 297, 1960).

PALYUSIK, M. (Institute of Epizootology of the Veterinary College, Budapest, Hungary): *Resistance to streptomycin of Chloramphenicol-treated E coli.*

In the intestinal tract of pigs treated with chloramphenicol certain *E. coli* strains became resistant not only to chloramphenicol but also to streptomycin. Chloramphenicol treatment of *E. coli in vitro* similarly enhanced streptomycin resistance.

GLÁZ, E., BOHUS, G. and SCHEIBER, E. (Institute of Pharmacology, University Medical School, Budapest, Hungary): *Antibiotic effect of Basidiomycetes on resistant bacteria and fungi.*

The antibiotic action of 120 Basidiomycetes against *St. aureus*, *E. coli*, *Pr. vulgaris*, *Ps. pyocyanea* and *C. albicans* was investigated. The *St. aureus* and *E. coli* strains were resistant to the 4 antibiotics in commonest use. Of the Basidiomycetes 24 inhibited *St. aureus*, 9 inhibited three or four of the bacteria mentioned above and further 7 inhibited *C. albicans*.

Isolation of the antibiotic substances, and determination of nutritional requirements of the antibiotic producing strains are in progress.

BALÁZS, V. and CSERHÁTI, I. (Department of Medicine, University Medical School, Szeged, Hungary): *Dynamics of developing resistance to streptomycin during streptomycin treatment of urinary infections.*

In patients with urinary infection streptomycin treatment was observed to fail more frequently than therapy with other antibiotics. To elucidate the cause of this phenomenon, 25 patients suffering from urinary *E. coli* infection were examined for living germ count, the relative number of resistant colonies and the degree of resistance several times before and during streptomycin treatment. Four types of reaction to streptomycin administration have been observed.

A kiadásért felel az Akadémiai Kiadó igazgatója

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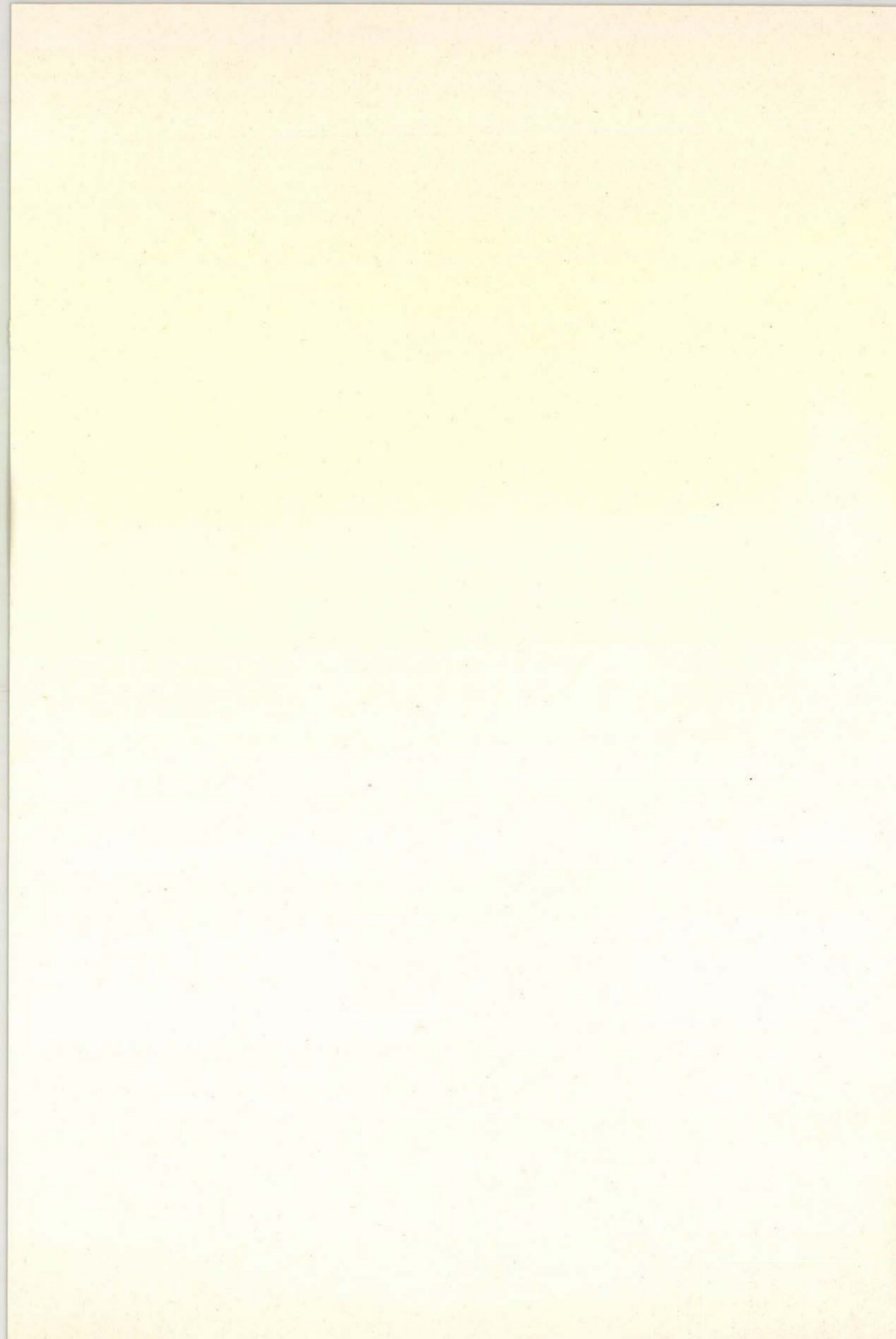
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ANTIGENICITY AND STABILITY ON STORAGE OF SALK TYPE VACCINES PRODUCED IN HUNGARY AND ABROAD

By

P. FÖLDES, HELEN SZERI and SUSANNA BÁNOS

Institute of Microbiology, University Medical School, Budapest

(Received November 7, 1959)

Summary. The stability on storage at room temperature of a Salk type vaccine prepared in Hungary and used in the epidemic of 1959 was examined. No decrease in antigenicity occurred on exposure to room temperature for a fortnight so that such storage must be considered to involve no damage. Both the Hungarian and a foreign polio vaccine preparation used in the 1959 epidemic were found to meet the minimum requirements when checked for their antigenic activity.

Mass vaccination against poliomyelitis has been carried out in Hungary since 1957. This involved the problem of appropriate storage during shipment and distribution.

The importance of appropriate storage was particularly emphasized by Austrian authors at the Semmering conference in 1958. It was said that the Salk vaccine produced in Austria exhibited a remarkable loss of antigenicity after 24 hours' storage at room temperature. This fact has been emphasized also in the directions supplied with the vaccine.

The results published by PERKINS *et al.* [1] from England were more favourable. As a loss was found in none but the type 1 component after 40 days' storage at 21° C, these authors took storage at room temperature for no longer than 1 week to be permissible.

The present studies were performed because it was thought improbable that a formalinized vaccine should be as heat labile as reported by others and because data were needed concerning the stability of the preparation used for mass vaccinations in the country where there is no guarantee for appropriate storage.

Materials and methods

The Hungarian vaccine examined was produced by the *Polio Vaccine Laboratory of the Virus Department of the State Institute of Hygiene, Budapest*, for use during the 1959 epidemic. The antigenic value of vaccine samples stored for a fortnight in a refrigerator was compared to samples stored at room temperature (between 21° and 23° C) during the same period. As a control, the antigenicity of the polio vaccine "106-3" produced abroad and stored in a refrigerator was also estimated.

Antigenicity was determined by the method suggested by SVEN GARD [2]. Serial tenfold dilutions of the material were inoculated to 8 guinea pigs each. After an appropriate period the animals were bled and their sera checked by the neutralization test to determine the highest dilution of antigen giving rise to a positive immune response. The negative loga-

rithms of these dilution endpoints are designated as "antigen extinction". When determined under the same conditions, the higher the extinction, the higher the antigenic value of the vaccine examined.

The neutralization tests were carried out in human embryonic fibroblast cultures. The amount of virus used was 100 CPD₅₀ with all the three types.

Results

Extinction values obtained for the Hungarian and the foreign Salk type polio vaccines are presented in *Table I*.

Table I

Antigen extinction values of a Hungarian and two foreign vaccines

Components	Hungarian vaccine stored		Foreign vaccine stored in refrigerator	
	in refrigerator	at room temperature	106-3	69-3
Type 1	2.34 (2.30)	2.40	2.55	(0.90)
Type 2	2.50 (>3)	2.68	2.55	
Type 3	2.34 (>3)	2.00	3.38	

The figures given in the second and the third column are the extinction values for the Hungarian vaccine. There was no significant difference between the values obtained for any of the three type components. Figures in brackets are those obtained by the *State Control Laboratory* during the official control of the vaccine.* The fitting of the two results was remarkable as the tests were carried out in different laboratories, under different conditions and at different points of time. It might be of interest that data obtained for the type 1 component fitted particularly well. In the fourth column the extinction values of the foreign vaccine preparation "106-3" are presented. It can be seen that the Hungarian preparation was not inferior particularly in respect of type 1 and 2 components. The extinction value of the type 1 component of another foreign vaccine was strikingly low, as shown in the fifth column. This latter preparation was used during the 1957 epidemic in Hungary.

The detailed titration results are presented in *Table II*.

In *Table III* the minimum requirements for the guinea pig immune response as determined by GARD [4] are presented. According to his comparative studies, to obtain 80 per cent positive immune response in triple negative children the minimum extinction values in guinea pigs should be 1.91, 0.88, and 2.10 for types 1, 2, and 3, respectively.

* We are indebted to DR. F. FORNOSI, head of the laboratory, for kindly supplying these data.

Table II
Results of extinction value titrations

Components	Dilution	Vaccine		
		H (i)	H(r)	106-3
Type 1	CC	8/8	8/8	7/7
	10 ⁻¹	8/8	7/8	7/7
	10 ⁻²	6/8	6/8	5/8
	10 ⁻³	0/8	1/8	2/5
	10 ⁻⁴	0/8	0/8	0/5
Type 2	CC	8/8	8/8	7/7
	10 ⁻¹	8/8	6/8	7/7
	10 ⁻²	5/8	6/8	6/8
	10 ⁻³	3/8	3/8	1/5
	10 ⁻⁴	0/8	0/8	1/5
Type 3	CC	8/8	8/8	7/7
	10 ⁻¹	6/8	8/8	7/7
	10 ⁻²	6/8	4/8	8/8
	10 ⁻³	0/8	0/8	4/5
	10 ⁻⁴	0/8	0/8	0/5

Explanations :

H(i) Hungarian vaccine stored in ice box

H(r) Hungarian vaccine stored at room temperature

106-3 foreign vaccine stored in refrigerator

The denominator shows the number of treated, the numerator the number of positively reacting animals.

Table III
Minimum requirements according to Gard

Type	Extinction
1	1.91
2	0.88
3	2.10

Discussion

Our data concerning the stability of the Hungarian polio vaccine on storage do not suffice for establishing the statistical significance of the differences obtained. The results published by LYCKE [3] might be of help in evaluating our results. He performed 60 parallel determinations by GARD's method and found that, as the standard deviation of the extinction values was $1/3 \log$

(0.349), differences higher than 1 log unit might be regarded significant. Accepting this we may conclude that our data did not reveal any significant difference in the extinction values of the two differently stored vaccine samples.

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Address of the authors:

PÁL FÖLDES, ILONA SZERI, ZSUZSANNA BÁNOS

Institute of Microbiology, University Medical School, Hőgyes E. u. 9, Budapest IX, Hungary.

THE VIRUS SPECTRUM OF A CELL LINE DERIVED FROM MONKEY KIDNEY CELLS

By

M. SIMON and AGNES JANCsó

State Institute of Hygiene, Budapest

(Received November 9, 1959)

Summary. The virus spectrum of a continuous line (PM) of monkey kidney cells was investigated. The cell line is now in its 40th passage. Its multiplication rate agrees with that of the primary monkey kidney cultures. Regression in growth probably caused by environmental factors occurred at the 20th, 30th and 38th passage of the cell line. Transformation did not occur. In comparative experiments carried out with laboratory strains and clinical material the new line allowed isolation of enteroviruses about as successfully as primary monkey kidney cell cultures.

The laboratory diagnosis of enterovirus infections is based chiefly on primary monkey kidney (MK) cell cultures in spite of the economical and other reasons suggesting the use of continuous MK cell lines. The latter, however, should be similarly sensitive to viruses, and their sensitivity should not be subject to substantial changes during serial passages. Since the pioneer work of WESTWOOD *et al.* [1, 2] only few reports [3, 4] have been published on the cultivation of new MK cell lines.

One of us in 1959 succeeded in cultivating a MK cell line termed PM [5]. In the following we shall present data (i) on the growth characteristics of the cell line PM, and (ii) on its sensitivity to viruses compared with that of primary MK cell cultures.

Materials and methods

Cultivation of the cell line PM. The cell line was derived from two tube cultures of the kidney cells of 5 healthy cynomolgus monkeys. The circumstances of isolation have been described elsewhere [5]. The cell line has been cultivated for more than a year covering 40 passages. The cultures used in the investigations reported here had undergone from 16 to 38 passages *in vitro*. The growth medium used consisted of 10 per cent calf serum, 45 per cent Parker's medium 199, and 45 per cent Hanks' solution containing 0.5 per cent lactalbumin hydrolysate. Penicillin, 100 units/ml, and streptomycin, 50 μ g/ml, were included in both this medium and the other media employed.

Serial cultivation was carried out in Roux culture flasks. The initial cell count was 4 million. The culture was confluent by the 7th to 9th day of incubation without changing the medium. Tube cultures were prepared by trypsinization of the confluent cultures. Trypsin was used at 0.25 per cent concentration. The initial cell count was from 70 000 to 80 000/tube. Tubes were incubated for 6 or 7 days at 37° C before inoculated. As maintenance solution Parker's medium 199 was used for the virus titrations: in the isolation experiments the growth medium was used.

Primary MK cell cultures. These were prepared as described by YOUNGNER [6]. Stationary cultures were used. The growth medium consisted of 98 per cent Hanks' solution with 0.25 per cent lactalbumin hydrolysate, and 2 per cent calf serum. The initial cell count was

200 000 cells/tube. The tube cultures were inoculated after one week's incubation at 37° C. The infected cultures were maintained in Parker's medium 199.

HeLa cells were cultivated as recommended by BÉLÁDI [7].

Viruses. The poliovirus strains Mahoney, MEF₁ and Saukett were those used in the production of Salk vaccine at the State Institute of Hygiene, Budapest. Sabin's type 1 attenuated LSc 2ab strain was kindly supplied by Prof. M. P. CHUMAKOV (*Moscow*). The ECHO, Coxsackie A, Coxsackie B and adenovirus strains originated from the collection of the State Institute of Hygiene, Budapest. The adenovirus strains were cultivated in HeLa cells, the ECHO, Coxsackie B and poliovirus strains in primary MK cultures. The Coxsackie A strains were obtained as 10 to 20 per cent suckling mouse muscle suspensions and used in 1:10 dilution.

Isolation of viruses. Faecal samples, in several cases CFS or throat washings, were treated with antibiotics and centrifuged; each material was simultaneously inoculated into primary MK and PM cultures as recommended by Dömök *et al.* [8]. For the identification of viruses, the standard sera of the State Institute of Hygiene, Budapest, were used.

Tests for sensitivity to viruses. To obtain preliminary information on the susceptibility to viruses of the cell line PM, 4 tube cultures were inoculated with low dilutions of each of the standard viral suspensions listed above. The viruses showing cytopathogenic effect were titrated in parallel in PM and primary MK cultures (enteroviruses), or in PM and HeLa cultures (adenoviruses). Tenfold dilution series were prepared in Parker's medium 199. Each dilution of the polioviruses was inoculated into 5 PM and 5 MK tubes, of the other viruses into 3 PM and 3 MK or HeLa tubes. The tubes were incubated with the inoculum (0.1 ml) for 1 hour before adding 0.9 ml Parker's medium 199. Incubation at 37° C was then continued the tubes lying horizontally, without changing the medium for 8 or 9 days in the case of polioviruses, and for 10 to 12 days with the rest. Titre was calculated according to the REED—MUENCH formula [9] and expressed in TCID₅₀/ml.

Experimental

Growth characteristics of cell line PM. Cell line PM was obtained from two tube subcultures (PM1 and PM2) of a pool of the trypsinized kidneys of 5 cynomolgus monkeys at about the 100th day of cultivation. The subcultures were taken 69 and 83 days, respectively, after primary culture. Transformation was



Fig. 1. PM subculture incubated for 3 days. May—Grünwald and Giemsa's stain

not observed. The cultures consist of epitheloid cells resembling primary MK cells in shape and size. The cells like those in the non-transformed MK1 line of WESTWOOD *et al.* [1] are delicate in structure and their cytoplasm is rather light. In the early stage of cultivation a lattice of cells can be seen (*Fig. 1*). Later, especially in the PM1 subcultures, the cells growing over the spaces of the lattice tend to form fan-like patterns (*Fig. 2*) also characteristic of the MK1 cultures. The morphology of certain PM2 subcultures is somewhat different. The cell sheet developed as a result of oriented growth, like that in the primary MK cultures, often shows a bundle-like structure (*Fig. 3*). Never-

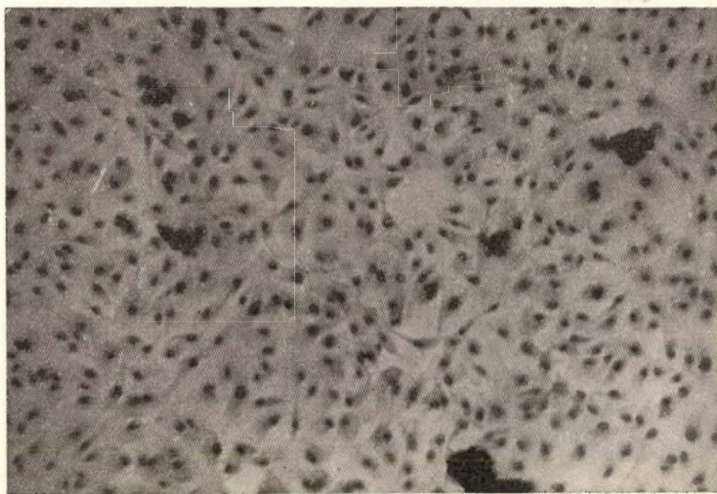


Fig. 2. PM subculture incubated for 7 days. Note fan-like appearance of growth.
May—Grünwald and Giemsa's stain

theless, the slight morphological difference between PM1 and PM2 cultures allows to regard the two cell lines as a single one. The multiplication rate of the cell line agrees with that of primary MK cultures. Roux flasks with an initial cell count of 4 million yield about three times as many cells 7 days later.

Subcultures were usually made every week. The medium was not changed in the meantime. If instead of making subcultures the medium was changed, the cultures could be maintained for a month without any apparent sign of degeneration. The PM cells adhere to the glass rather firmly. Viable cells are not released unless incubated at 37° C with 0.25 per cent trypsin or with 1 : 5000 diluted versene for 30 to 60 minutes.

It was attempted to cultivate the cell line in different media. One of these was composed of 10 per cent calf serum and 90 per cent Parker's medium 199, another of 10 per cent calf serum and 90 per cent Hanks' solution contain-

ing 0.25 per cent lactalbumin hydrolysate. In the latter medium the cells showed degeneration as early as in the second passage and could not be cultivated in the third subculture. In the medium containing Parker's 199 the cells also underwent some change. They became elongated and so their epitheloid character changed into a fibroblast-like appearance.

The multiplication rate gradually diminished. The cell line was cultivated in this medium for about 6 months, but subcultures could not be made more frequently than once every three weeks.



Fig. 3. PM subculture incubated for 7 days. Note bundle-like growth. May—Grünwald and Giemsa's stain

At its 20th, 30th and 38th passage the cell line PM showed some morphological changes accompanied by a decrease in the multiplication rate. Enlarged, elongated, pleomorphic cells appeared, and the cytoplasm, originally free of granules, became granulated (*Fig. 4*). With every such change a considerable proportion of the cultures were lost. Another part of the cultures had regained the original morphology and grew at the normal rate. The cause of the regression periods is discussed below.

The sensitivity of the cell line PM to viruses. Large inocula of each of the poliovirus strains Mahoney, MEF1, Saukett and the attenuated strain LSc 2ab, of types 1 to 12 and 14 to 19 of ECHO viruses, of types 2 to 5 of Coxsackie B and of the Coxsackie A9 virus exerted a cytopathogenic effect in the PM cultures, whereas neither of the Coxsackie A types 4, 11, 12, 13, 15 and 16 caused any such effect. PM and primary MK cultures inoculated with 1000 or more TCID₅₀ of poliovirus showed cytopathic changes as early as 18 hours after inoculation; a delay of one or two days was observed in the PM cultures

if the inoculum was more dilute. The cultures were therefore observed up to the 9th day after infection.

It was attempted to propagate poliovirus serially in PM cultures. The Mahoney strain was propagated in 10, the Saukett strain in 5 consecutive passages. The cumulative dilution exerting cytopathogenic effect was 10^{-43} and $10^{-24.2}$, respectively.

Table I

Comparative titration in PM and primary monkey kidney (MK) cultures of representative strains of poliovirus

Number of passages in PM cultures	Type 1 (Mahoney)		Type 2 (MEF ₁)		Type 3 (Saukett)	
	MK	PM	MK	PM	MK	PM
0	6.6*	5.0	7.5	6.6	7.5	7.6
1	7.2	6.2	6.2	5.5	6.6	6.5
5	7.0	7.4	NT**	NT	7.5	7.2
10	6.7	6.0	NT	NT	NT	NT

* log TCID₅₀/1.0 ml

** Not tested

Comparative titrations of poliovirus preparations in PM and primary MK cultures are demonstrated in *Table I*. The cell line PM was somewhat less sus-

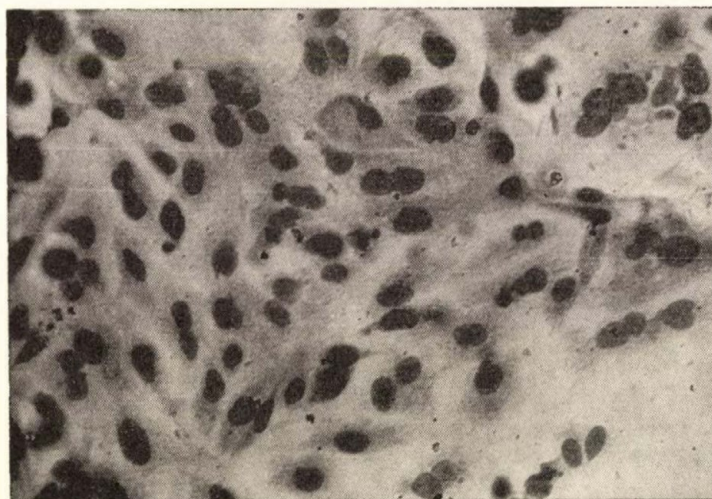


Fig. 4. Cell line PM in the regression period. Passage 30. Subculture incubated for 10 days. Note giant cells some of which are multinucleated. May—Grünwald and Giemsa's stain

ceptible to poliovirus 1 and 2 infections than the primary MK cultures. Such difference was not observed in the case of type 3 poliovirus.

In the MK cell line "MS" of KANDA and MELNICK [3] virulent poliovirus strains grew well whereas attenuated strains barely. We titrated SABIN's LSc 2ab attenuated type 1 poliovirus, and a wild type 1 strain in primary MK cultures and PM cultures comparatively. All the infected cultures were incubated at 37° C. The results are shown in *Table II*.

Table II
*Comparative titres in PM and primary MK cultures
of a virulent and an attenuated strain of type 1 poliovirus*

Virus	Titres expressed in log TCID ₅₀ /ml as established in	
	primary MK	PM
Virulent	6.5	6.0
Sabin's attenuated	6.0	5.5

The quotient $\frac{\text{titre in MK}}{\text{titre in PM}}$ was the same for the two strains tested. Our isolation experiments also showed that our cell line is susceptible to attenuated poliovirus strains.

Certain Coxsackie and ECHO virus strains were also studied (*Table III*). In several instances, marked by* in *Table III*, the titrations in PM and MK cultures were not done simultaneously; titration in PM cultures was done about 6 months later.

Table III
Comparative titrations of viruses in different cell cultures

Virus	Titres expressed in TCID ₅₀ /1.0 ml as established in		
	primary MK	HeLa	PM
	cell cultures		
Coxsackie B3	7.0*		7.5
ECHO 1	6.7		6.5
ECHO 2	7.5*		7.0
ECHO 9	7.5*		5.0
Adeno 5		6.5	2.5
Adeno 6	2.0	7.0	2.5
Adeno 7		6.6	2.0

* Titration was performed 6 months earlier in MK cultures than in PM cultures

During that six-month period the viral preparations, usually stored in the frozen state, were once or twice thawed and frozen again. In spite of this, Coxsackie A9 was the only virus giving significantly lower titre in PM cultures

than in primary MK cultures. The three adenovirus strains (types 5, 6 and 7) gave 4 to 5 $\log_{10}$ units higher titres in HeLa cells than in PM cultures.

Isolation of viruses in PM cultures. A hundred and twenty specimens, of these 107 faeces, 10 CSF and 3 throat swabs, were examined in PM and primary MK cultures, in simultaneous experiments. Forty-one specimens originated from patients, 79 from children fed with SABIN's live attenuated vaccine. Virus was isolated in primary cultures from 37, in PM cultures from 36 specimens. Thirty-three specimens yielded virus in both types of culture, three only in primary cultures, whereas two only in the PM cells. Of the strains isolated in primary cultures 31, of those isolated in PM cells 30 have been identified, by neutralization tests carried out in both types of culture. The distribution by type of the isolates is demonstrated in *Table IV*.

Table IV

Comparative isolation experiments in PM and primary MK cultures

Specimen	Number of specimens	Specimens yielding virus in		Type of the strains	Isolated in	
		MK	PM		MK	PM
Faeces from patients	28	6	6	Polio 1	4	4
				unidentified	2	2
Faeces from children fed with Sabin's vaccine	79	30	29	Polio 1	13	13
				Polio 2	3	3
				Polio 3	3	3
				Polio 1 & 2	1	—
				Polio 1 & 3	2	2
				Polio 2 & 3	1	1
				Polio 1 & ECHO	1	1
				Polio 2 & ECHO	—	1
				Polio 3 & ECHO	1	1
				ECHO	2	1
				unidentified	3	3
CSF from patients	10	—	—			
Throat swab from patients	3	1	1	unidentified	1	1

Table IV shows that in the laboratory diagnosis of enteroviruses the cell line PM proved to be about as susceptible as the primary MK culture. In these experiments too cytopathic changes appeared in the PM cultures one or two days later than in the primary cultures. The morphological changes in the primary and PM cultures were identical. Faecal specimens toxic for primary cultures proved in the most instances toxic also for the PM cultures. Nevertheless, somewhat fewer suspensions were found to be toxic for the latter.

Discussion

Several authors [3, 10] have applied continuous MK cell cultures in virus research. The practical use of the transformed MK2 line of WESTWOOD *et al.* is widely known. On the other hand, we have failed to find in the literature detailed studies on the susceptibility to viruses of non-transformed cell lines.

Our findings that the cell line PM has a virus spectrum comparable with that of the primary MK cultures, and that its susceptibility to viruses was unchanged between its 16th and 38th passages, suggest that this cell line is applicable in the isolation of enteroviruses from clinical specimens.

It should be noted, however, that between its 16th and 38th passages the cell line underwent three regression periods accompanied by morphological changes. Although the exact cause of the regression has not been revealed, we tend to attribute it to environmental factors. One of the regression periods immediately followed the introduction of a new batch of growth medium. As soon as the changes had been noted, the medium was substituted in certain tubes for an old batch of the same medium. Regeneration took place in these flasks sooner than in those to which the new batch was added again.

On the other hand, the regression periods of the cell line PM remind us of the phenomenon called pseudotransformation by WESTWOOD and TITMUSS [2]. Like the MK3 line of these authors, our PM line also shows certain signs characteristic of transformation, *viz.* atypical mitoses and multinucleated cells neither of which occur in primary MK cultures.

Since the regressive phenomena to some extent resembled cytopathic changes caused by viruses, an attempt was made to subculture the hypothetical virus. Ten primary monkey kidney and ten rabbit kidney cell cultures were inoculated with the nutrient medium of each of two cultures showing degeneration. Cytopathic changes did not appear.

Based on the satisfactory results of isolation experiments and regarding its slow growing, the PM line might be used in isolation of enteroviruses in laboratories having not too great a number of specimens to be examined. The morphological unstability of the cell line and the regressive phenomena make it imperative to check its susceptibility to viruses from time to time.

Finally, it is noteworthy that 10 per cent calf serum in the maintenance medium did not inhibit viral multiplication. The same batch of serum was used throughout.

Acknowledgements. We are indebted to Drs I. DÖMÖK and A. KOCH for their helpful advice and criticism.

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Address of the authors:

MIKLÓS SIMON, Lehel tér 2/d, Budapest XIII, Hungary

ÁGNES JANCsó, State Institute of Hygiene, Gyáli út 2-6, Budapest IX, Hungary.

A RAPID METHOD FOR THE INVESTIGATION OF RAFFINOSE FERMENTATION BY YEASTS*

By

E. K. NOVÁK

Mycological Laboratory, State Institute of Hygiene, Budapest

(Received November 26, 1959)

Summary. A rapid method has been elaborated for determining the raffinose fermentation quotient of yeasts. The method is more rapid, simple, and more accurate than are earlier procedures; it is based on establishing the fermentation quotient from the chromatogram of fermented raffinose solution. In the fermented fluid, the presence of melibiose indicates 1/3 fermentation of raffinose, that of galactose 2/3 fermentation, while the absence of sugar furnishes proof of 3/3 fermentation of raffinose.

A new technique has been devised for the prompt visualization of sugars in the chromatogram without heating.

The method allows reliable determination of the quotient of raffinose fermentation in one hour from the end of fermentation.

Serial raffinose breakdown and utilization have been demonstrated by chromatography in the fermentation of raffinose by *Saccharomyces carlsbergensis*.

The raffinose fermentation *i. e.* the fermentation quotient of yeasts is an important taxonomical property [1]. The trisaccharide raffinose consists of three monosaccharide components — galactose, glucose, and fructose (*Fig. 1*). The capacity of any kind of yeast for fermenting raffinose at the ratio of 1/3, 2/3, or 3/3 depends on the particular enzymes it incorporates of those required for breakdown, while one group of the species is unable to ferment that sugar. The three processes of raffinose fermentation are outlined in *Fig. 2*.

Various methods have been developed for determining the fermentation quotient, but they are either too elaborate, or inaccurate, or very complicated. KLUYVER calculated the quotient on the basis of carbon dioxide volumes obtained by the aid of VAN ITERSON—KLUYVER's fermentometer [2]. This method is inaccurate owing to the volumetric measurement of gas. VICKERHAM first computed the quotient from the second fermentation brought about in fermentation fluid re-inoculated with *Saccharomyces carlsbergensis* — fermenting raffinose at the ratio of 3/3 — following previous fermentation of 4 per cent raffinose solution with the strain under review [3]; later he determined the fermentation of melibiose by the tested strain for calculation of the quotient [4]. In this case, the first method is lengthy as a result of two consecutive processes of fermentation, while the second procedure involves double work due to the introduction of melibiose. SKINNER and BOUTHILET produced melibiose

* Lecture at the Meeting of the Mycological Section of the Hungarian Microbiological Association, June 9, 1959.

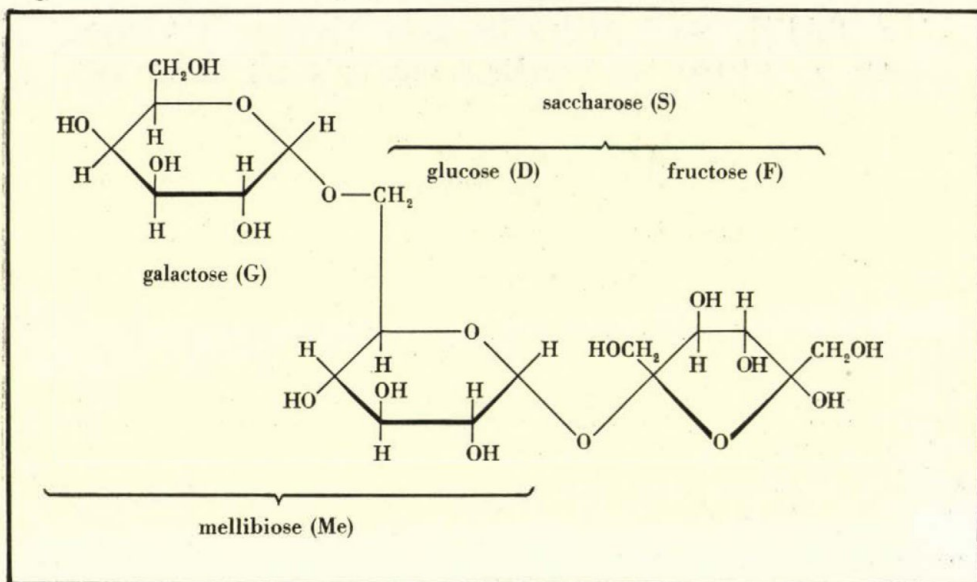


Fig. 1. The structure of raffinose

Type	Process
1/3	$\begin{array}{c} \text{invertase} \\ \downarrow \\ \text{G-D-F} \longrightarrow \text{G-D} + \text{F} \longrightarrow \text{C}_2\text{H}_5\text{OH} + \text{CO}_2 \\ \text{raffinose} \quad \text{mellibiose} \quad \text{fructose} \end{array}$
2/3	$\begin{array}{c} \text{invertase} \\ \downarrow \\ \text{G-D-F} \longrightarrow \text{G-D} + \text{F} \longrightarrow \text{C}_2\text{H}_5\text{OH} + \text{CO}_2 \\ \text{raffinose} \quad \text{mellibiose} \quad \text{fructose} \\ \downarrow \leftarrow \alpha\text{-galactosidase (mellibiase)} \\ \text{G} + \text{D} \longrightarrow \text{C}_2\text{H}_5\text{OH} + \text{CO}_2 \\ \text{galactose} \end{array}$
3/3	$\begin{array}{c} \text{invertase} \\ \downarrow \\ \text{G-D-F} \longrightarrow \text{G-D} + \text{F} \longrightarrow \text{C}_2\text{H}_5\text{OH} + \text{CO}_2 \\ \text{raffinose} \quad \text{mellibiose} \quad \text{fructose} \\ \downarrow \leftarrow \alpha\text{-galactosidase (mellibiase)} \\ \boxed{\text{G} + \text{D}} \longrightarrow \text{C}_2\text{H}_5\text{OH} + \text{CO}_2 \\ \text{galactose} \quad \text{glucose} \end{array}$

Fig. 2. The mechanism of raffinose fermentation

solution [5] from raffinose with live *Saccharomyces cerevisiae* var. *ellipsoideus* — fermenting raffinose at the rate of 1/3. This melibiose, which in solution may be used immediately for fermentation, is fermented in a fortnight, more rapidly than VICKERHAM's melibiose, a chemical product taking three or four weeks to ferment. Moreover, biologically developed melibiose solution, when evaporated to half volume, undergoes fermentation in a week. Notwithstanding its relatively quicker operation, this method has retained the faults of VICKERHAM's procedure, and the cost of additional work. The appearance of reducing sugars has been demonstrated by VAN UDEN with BENEDICT's reagent in raffinose solution fermented with the strain being tested [6]. By this procedure only 3/3 fermentation is distinguished from 2/3 and 1/3 fermentation, because in the case of the former no sugar is left over, whereas in the latter case reducing sugar remains in the solution. In addition, 2/3 and 1/3 fermentation can be differentiated only by the aid of melibiose fermentation.

Upon inspection of the processes presented in Fig. 2 it is obvious that after 1/3 fermentation the fluid contains melibiose, after 2/3 fermentation it contains galactose, while following 3/3 fermentation there is no residual sugar. (In the figure the names of residual sugars have been underlined.) Thus, chromatography of fermentation fluid samples taken at the end of the process and sprayed with sugar reagent would make it possible to read the fermentation quotient from the chromatogram.

Methods and results

The following method has been devised for the demonstration and identification of sugar present in the solution at the end of fermentation.

0.5 per cent peptone water, containing 4 per cent raffinose, is inoculated with the culture to be tested. Fermentation takes a few days, depending on the inoculum. When the process is over, 20 μ l of the fermentation fluid are transferred with a micro-pipette or a simple glass capillary onto a circular chromatographic filtre paper 12.5 cm in diameter. Amounts of 20 μ l of 2 per cent raffinose, melibiose, galactose, and glucose solutions each are run simultaneously as controls. The paper is inserted between the lids of two 12 cm Petri dishes, and chromatogram is run in butanol/acetic acid/water 4 : 1 : 1 [7]. A small piece of tightly rolled filtre paper is stuck into the hole made in the centre of the filtre paper, to make contact with the solvent. Chromatography takes about 40 to 60 minutes, depending on the kind of paper employed (a medium dense paper of unknown origin has been found to be exceptionally suitable by ourselves). Subsequently the paper is dried, sprayed with 0.5 per cent benzidine dissolved in acetic acid, or with benzidine-trichloroacetic acid [8]; after mild drying it is placed for a few minutes into a cylinder filled with resin-free furfural steam, without heating. In a minute or two, the yellow spots appearing

on the bluish background indicate the sites of sugar. The reaction is given also by the non-reducing raffinose. The spots should be marked, because they change colour under storage.

Fig. 3 shows the chromatograms of fermentation fluid with a 2/3 raffinose-fermenting yeast (*Candida melibiosi*), and a 1/3 fermenting species (*Saccharomyces marxianus*), on the fourth day of fermentation, in an approximately semi-fermented condition. For phototechnical reasons these chromatograms were sprayed with benzidine-trichloroacetic acid and heated to 105° C for five minutes [8]. The R_f values are presented in Table I.

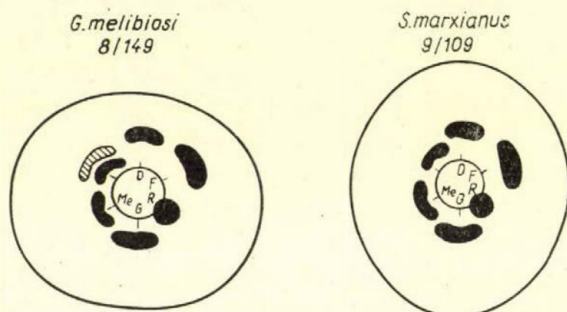


Fig. 3. Left side. Chromatogram of raffinose fermentation by 2/3 fermentator of raffinose (*Candida melibiosi*). Fermented raffinose solution in unmarked spot; in the other areas, starting from the top, clockwise, the controls, D standing for glucose, F for fructose, R for raffinose, G for galactose, Me for melibiose. Right side. Chromatogram of raffinose fermentation by 1/3 fermentator of raffinose (*Saccharomyces marxianus*). Materials as on left side

Table I

Materials	R_f^*	
Glucose (D)	0.304	(0.30—0.31)
Galactose (G)	0.267	(0.26—0.28)
Fructose (F).....	0.363	(0.36—0.37)
Melibiose (Me)	0.108	(0.10—0.12)
Raffinose (R).....	0.072	(0.07—0.08)

* R_f values are averages of ten experiments; extreme values in brackets.

The above method has been applied for the investigation of the time factor in the course of the fermentation of raffinose by a 1/3 fermenting species (*Saccharomyces cerevisiae* var. *ellipsoideus*) and a 3/3 fermenting species (*Saccharomyces carlsbergensis*).

Fig. 4 shows 1/3 fermentation, Fig. 5 demonstrates 3/3 fermentation; the gradual course of the fermentation process, and the serial progress of 3/3 fermentation are apparent.

As evidenced by the chromatogram, of the melibiose and fructose produced from raffinose fructose is used up first. There is no breakdown of melibiose as long as cellular needs are covered by the fructose. Hence melibiose accumulates at the expense of raffinose. The decomposition of melibiose begins only when raffinose has been exhausted or strongly reduced in quantity. Of



Fig. 4. Fermentation of raffinose by *Saccharomyces cerevisiae* var. *ellypsoides* (1/3 fermentator of raffinose) in relation to time.

Controls: at left margin, melibiose; at right margin, glucose and galactose. The figures on the starting line denote the time of collection of samples reckoned in hours from the onset of fermentation

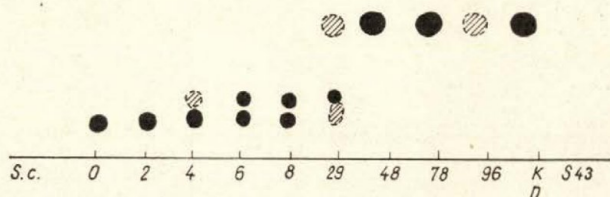


Fig. 5. Fermentation of raffinose by *Saccharomyces carlsbergensis* (3/3 fermentator of raffinose) in relation to time. Reproduction of chromatogram in drawing. The shaded spots are on the verge of demonstrability. At right margin, glucose as control. The figures on the starting line give the time of sample collection in hours. The spot with the lower R_f value is raffinose, the higher is melibiose, the highest is glucose or galactose

the produced glucose and galactose, only one is used up, most probably glucose, but owing to the closeness of R_f values this cannot be decided on the basis of the present chromatogram, as intensity of the spot increases at the beginning. The amount of sugar with an R_f corresponding to that of glucose or galactose begins to diminish when there no more melibiose is left.

The above facts present many problems connected with the strict order of sequence. These are, however, beyond the scope of this study. Some issues have been discussed in another paper [9]. Explanation of the phenomenon will in all likelihood be provided by differences in the activating energies of the enzymes involved, or competition of the substrates for common enzymes, possibly competition of substrate coenzyme complexes for common apoenzymes.

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Address of the author:

ERVIN K. NOVÁK

State Institute of Hygiene, Gyáli út 2/6, Budapest IX, Hungary

INHIBITION OF THE MULTIPLICATION OF INFLUENZA VIRUS BY FRANCIS INHIBITOR COUPLED TO ANILINE DERIVATIVES

By

I. HOLLÓS

State Institute of Hygiene, Budapest

(Received December 8, 1959)

Summary. Francis inhibitor was coupled to aniline derivatives. Coupling of some of the derivatives tested did not influence the interaction between inhibitor and influenza virus. Other coupled compounds, particularly alkylized ones, did not affect combination with the virus, but protracted, or even prevented liberation of the virus from the inhibitor virus complex. The latter type of coupled inhibitor behaved as a competitive antagonist not only of the adsorption of virus to erythrocytes, but also of viral multiplication in surviving tissues. One of the coupled preparations was tested in the allantoic cavity and was found to inhibit viral multiplication even if added 30 minutes after viral infection. In preliminary experiments inhibition of the early phase of infection was found to take place in the mouse lung.

HIRST in 1942 suggested that the erythrocyte receptor responsible for viral haemagglutination (HA) is an analogue of the virus receptor of the susceptible cell [1]. The role of the cell receptor in the multiplication of influenza virus is supported by some phenomena, namely, (i) a cyclic fluctuation in the amount of receptor substance during the process of multiplication [5]; (ii) the inhibition of both early and late phases of viral multiplication by the same chemical substance [6]; and (iii) the observation that influenza virus can be adsorbed to, and multiply in, cells the receptor apparatus of which is intact [4]. There are, however, some contradictory data [3] so that the role of receptor substance in viral multiplication is not yet clear.

A number of competitive inhibitors acting against HA by the influenza virus have been described [12]. However, neither of these inhibitors are capable of inhibiting viral multiplication significantly, since they are soon destroyed by the active virus [14, 15]. According to WOLLEY's concept [16] only those HA inhibitors are expected to inhibit viral multiplication which form a firmer complex with the virus than the cell receptor does.

The aim of the present work was to obtain such inhibitors. For this purpose Francis inhibitor was coupled to aniline derivatives.

Materials and methods

Francis inhibitor [7] was prepared by the method of HIRST [8]. Human blood plasma diluted 1:4 or 1.5 or 2.0 per cent solution of COHN's fraction IV or IV/4 was adjusted to the pH which a preliminary experiment had shown to be the optimum. This pH varied between 5.0 and 6.6 for the compounds tested. As solvent and diluent *M*/15 or *M*/60 phosphate buffer

was used. About 600 ml of the material was placed in a boiling water bath to heat it over 90° C for a few minutes. The precipitate formed was separated by centrifugation with a speed depending on the precipitate's dispersity. The supernatant, named "crude Francis inhibitor" was subjected to ethanol or acetone fractionation. The precipitates were dissolved in 1/5 or 1/10 volume of a pH 7 phosphate buffered saline and dialyzed against the same solution at +4° C for two days. The fine precipitate, if formed, was removed by centrifugation. The supernatant was the starting material. After coupling it to aniline derivatives the precipitate was removed, the material dialyzed for two days and subjected to ethanol or acetone fractionation, and subsequently again dialyzed against the buffered saline until the outer fluid had remained colourless. The uncoupled control preparation thus obtained was called "purified Francis inhibitor". The preparations were stored in a deep-freezer. Attempts were made to lyophilize them but the lyophilized material could never be dissolved completely. Each preparation was made up to the same volume and characterized by its HA inhibiting (HAI) titre.

As HA unit (HAU) the amount of viral haemagglutinin was chosen which was present in 1 ml of a viral suspension giving a partial HA. One HAI unit was that contained by 1 ml of a preparation causing partial inhibition if tested against a suspension of Lee virus in the indicator state containing 4 HAU per ml. Both HA and HAI were titrated according to TAKÁTSY [9].

Infectivity was titrated by HORVÁTH's roller drum micro technique [11].

The strains influenza A, PR8, influenza A₁ Budapest 4/49, influenza A₂ Singapore 1/57 (nonavid variant) and influenza B Lee were used. The viral preparations were stored in sealed phials in CO₂ ice.

Experimental

As demonstrated in preliminary experiments, the "purified Francis inhibitor" was completely inactivated by influenza virus at room temperature. The time of inactivation depended upon the strain and the relative concentration of virus and inhibitor.

Titration of the virus recoverable from inhibitor virus mixtures by adsorption to, and elution from erythrocytes. Experiment 1. Five ml of Budapest 4/49 allantoic fluid were added to the same volume of "purified Francis inhibitor". From the virus-inhibitor mixture kept at room temperature 1 ml samples were taken at intervals, mixed in an ice bath with 0.2 ml of a 5 per cent suspension of fowl erythrocytes, left standing for 15 minutes and then centrifuged. The erythrocyte sediment was resuspended in 0.5 ml saline, incubated at 37° C for 2 hours, and centrifuged. The supernatant was titrated for HA to determine the amount of virus recoverable from the erythrocytes. Virus without inhibitor was examined as control for each experiment. The results are illustrated in Fig. 1. The 0-hour titre of the control eluates are presented as 0-hour values.

In Fig. 1 the thick curve illustrates the mean titres from 6 experiments, the thin curves the extreme values. It is clearly seen that the amount of the recoverable virus suddenly dropped in the first hour, then increased gradually. By the sixth hour it approximated the 0-hour value.

Experiment 2. This experiment differed from Experiment 1 only in the concentration of the virus being a quarter of that in Experiment 1.

Fig. 2 shows a sudden decline in titre. In some of the experiments no virus could be demonstrated in one or two early samples but later the titre again attained the starting level.

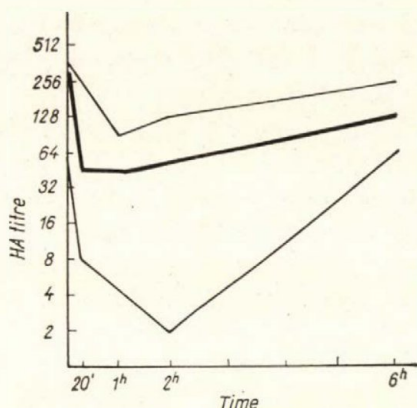


Fig. 1. Amount of virus recoverable from mixtures of virus and Francis inhibitor by adsorption to, and elution from, chicken erythrocytes. Budapest 4/49, 512 HAU/ml + Francis inhibitor, 64 HAIU/ml.

The thick line represents the geometric means from 6 experiments, the thin lines illustrate the extreme values

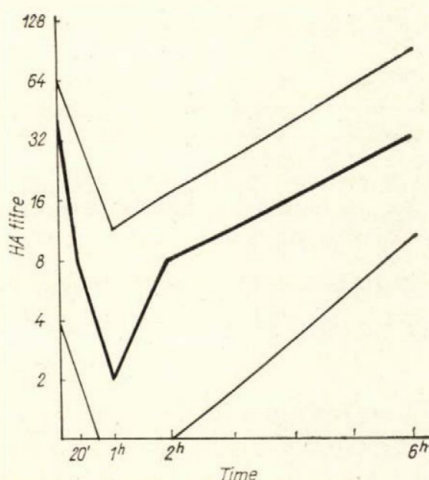


Fig. 2. Amount of virus recoverable from mixtures of virus and Francis inhibitor by adsorption to, and elution from, chicken erythrocytes. Budapest 4/49, 128 HAU/ml + Francis inhibitor, 64 HAIU/ml.

The thick line represents the geometric means from 6 experiments, the thin lines illustrate the extreme values

To stabilize its inhibitory effect, Francis inhibitor was coupled to compounds which were supposed to leave the polysaccharide component of the inhibitor unaffected. According to CURTAIN [10] it appeared the most promising to couple diazonium salts to the inhibitor's protein component.

Among the coupled preparations tested that coupled to diazotized p-phenylene diamine was the first showing a modified effect. This substance

was prepared by adding 3 mg p-phenylene diamine in 1 ml to 3 mg lyophilized Francis inhibitor dissolved in 1 ml. (As mentioned before, the lyophilized material could not be dissolved completely.)

Experiment 3. In this experiment the p-phenylene diamine-coupled inhibitor was examined in the same way as the purified Francis inhibitor was in Experiments 1 and 2. The results are shown in Fig. 3.

If a large amount of virus was added to the coupled inhibitor, as little as 1/32 of the viral haemagglutinin was recoverable after incubation for 35 minutes, but in the 6th hour its 1/3 could be detected again. If however, like

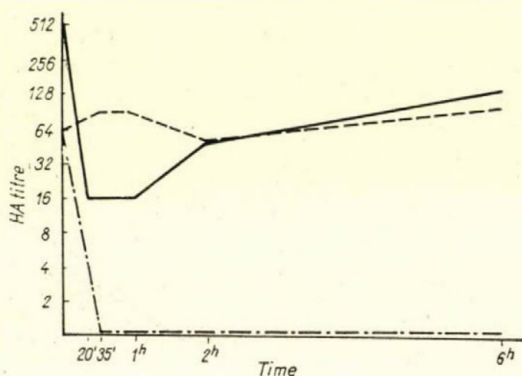


Fig. 3. Amount of virus recoverable from mixtures of virus and coupled inhibitor, by adsorption to, and elution from, chicken erythrocytes

- Budapest 4/49, 512 HAU/ml + Francis inhibitor coupled to p-phenylene diamine, 64 HAIU/ml
- - - - Budapest 4/49, 128 HAU/ml + Francis inhibitor coupled to p-phenylene diamine, 64 HAIU/ml
- . - . Budapest 4/49, 128 HAU/ml + p-phenylene diamine, 3 mg/ml

in Experiment 2, less virus was added, virus could not be detected in any of the samples taken during the 6 hours of the experiment. Thus the virus was bound firmer by Francis inhibitor coupled to diazotized p-phenylene diamine than by the uncoupled inhibitor.

In a special control experiment, also shown in Fig. 3, instead of the modified inhibitor an aliquot of a solution containing 3 mg p-phenylene diamine was added to an equal volume of the viral preparation used in the former experiments. No significant loss in HA activity was observed in any of the samples taken during the next 6 hours. The direct inhibiting effect of p-phenylene diamine was thus excluded. Besides, any free p-phenylene diamine must have remained in the discarded supernatants or dialyzed out in the course of fractionation.

The loss in viral haemagglutinin was stable in those experiments, too, in which Francis inhibitor was coupled to some other aniline derivatives, partic-

ularly to compounds with an alkyl group linked to the ring itself, or in which the H atoms of the second amino group had been substituted by alkyl groups. On the other hand, coupling to Cl derivatives, p-amino-methyl ketol, sulphonamide derivatives, p-amino salicylic acid, or procaine did not modify the effect.

In the following experiments it was attempted to demonstrate the existence of a competitive antagonism between the modified inhibitors and the surviving chicken embryonic tissue.

Inhibition of viral multiplication in chicken embryonic tissue. HORVÁTH's roller drum micro technique was used. The inhibitor was diluted with the nutrient fluid, and each dilution was measured in at least 8 tubes. Then the suspended tissue was added, and, finally, within 10 minutes, 10^3 , 10^4 or 10^5

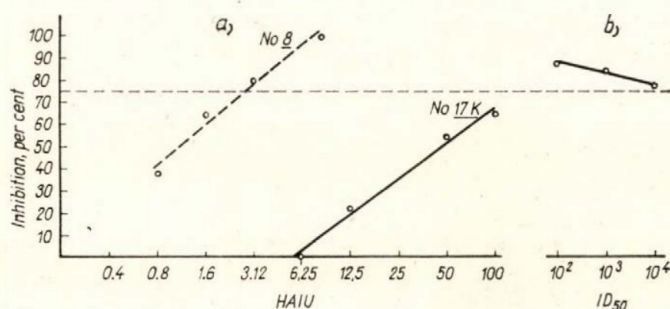


Fig. 4. Inhibition of multiplication of Lee strain in HORVÁTH's rolling drum

- a) Inhibition of 10^4 ID₅₀ by varying doses of inhibitor
 b) Inhibition of varying number of infective doses by 3.2 HAIU of preparation No. 8

ID₅₀ of virus. When the tubes had rolled for 72 hours, the contents were titrated for haemagglutinin. (The titre of the inoculum was always controlled simultaneously. The titre of the stock viral suspensions stored in dry ice was found rather stable. The Budapest 4/49 stock preparation was titrated for infectivity in each of 8 subsequent weeks. Scattering did not exceed 0.5 log unit.) In each experiment the average HA titre in the vials containing no inhibitor was regarded as 100 per cent.

Experiment 4. Fig. 4 illustrates an experiment in which Francis inhibitor coupled to diethyl-p-phenylene diamine (preparation No. 8) was tested against 10^4 ID₅₀ of Lee virus.

0.8 HAIU of the inhibitor was just enough to exert a demonstrable inhibition, and in the presence of 6.25 HAIU no virus growth could be demonstrated (100 per cent inhibition). The relation between log dose and inhibition per cent appeared to be approximately linear.

In the same experiment preparation 17K was also examined. This was an uncoupled Francis inhibitor which had undergone all the damaging procedures of coupling, which were mainly of oxydative character. 6.25 HAIU of

this preparation did not exhibit inhibition, and even 100 HAIU were not sufficient to cause total inhibition. The slopes of the two straight lines in *Fig. 4* are approximately the same. It is also shown that the amount of virus had no significant influence on the percentual inhibition. The dotted horizontal line represents 75 per cent inhibition.

Experiment 5. *Fig. 5* illustrates an experiment similar to Experiment 4, but with the strain influenza A₂ Singapore 1/57 (non-avid variant).

As shown in *Fig. 5*, the log dose-inhibition curves both of preparation No 8 and 17K are steeper, and both preparation proved to be somewhat less active against the Singapore strain than against the Lee strain. The similar

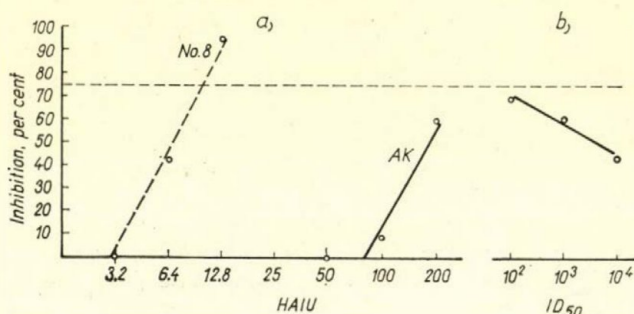


Fig. 5. Inhibition of multiplication of Singapore strain in HORVÁTH's rolling drum
 a) Inhibition of 10⁴ ID₅₀ by varying doses of inhibitor
 b) Inhibition of varying number of infective doses by 6.4 HAIU of preparation No. 8

curves of the strains influenza A PR8 and influenza A1 Budapest 4/49 fell between the corresponding curves of the Singapore and the Lee viruses.

To determine the relative potency of the modified inhibitors, different concentrations were tested, the highest and lowest of which caused 100 per cent and no inhibition, respectively. It is noteworthy that certain sub-threshold doses of the inhibitors, including of the uncoupled preparation 17K, generally showed a slight but definite stimulation of viral growth.

Table I shows the comparative data of the most active 3 preparations, based on the dose of each preparation causing 75 per cent inhibition as computed by interpolation. For the Budapest 4/49 strain this dose was determined in 5 experiments. The coefficient of variation as multiplied by Student's value was ± 55 per cent. As the 3 most potent preparations in a number of experiments proved to be about 100 times as potent as preparation 17K, the enhancing effect of coupling on the inhibiting activity of Francis inhibitor may be regarded as significant. It could not be decided exactly, which of the three preparations was the most potent, for potency seemed to depend also on the strain against which the compound was tested. With high doses of preparation

Table I

Doses of Francis inhibitor preparation causing 75 per cent inhibition (D_{75}) against 10 000 ID₅₀ of 4 different strains

Preparation No.	Compound coupled Chemical name	D_{75} against			
		Lee	PR 8	Bp 4/49*	Sing**
3	p-ethyl aniline	0.75	7.0	0.8	11.6
8	diethyl-p-phenylene-diamine	3.0	7.6	3.2	10.2
1	o-methyl aniline	4.0	10.2	4.8	5.5
17 K	None (treated Francis inh.)	110	204	204	>204
0	None (purified Francis inh.)	200	>300	>200	>200

* Budapest 4/49

** Singapore 1/57 (nonavid)

17K, 75 per cent inhibition was attained against 3 of the 4 strains whereas only the Lee strain was inhibited to such an extent with the highest doses of "purified Francis inhibitor". Usually, more inhibitor was needed to inhibit the growth of the Singapore strain than of any of the other three, presumably owing to differences in affinity to receptor substances [20].

Table II

*Doses of coupled Francis inhibitor preparations causing 75 per cent (D_{75}) inhibition of multiplication of Singapore (non-avid) strain**

Preparation No.	Compound coupled Chemical name	D_{75} in HAIU
15	o-anizidine tetrazonium chloride	12.7
6	p-phenylene diamine	16.4
19	o-p-propyl aniline	23.8
18	o-propyl aniline	28.1
2	o-aethyl aniline	42.6
10	o-propyl-p-phenylene aniline	67
12	tryptaflavine	70
4	o-p-dimethyl aniline	81
14	o-azotoluene diazonium chloride	100
7	dimethyl-p-phenylene diamine	160

* Inoculum, 10 000 ID₅₀

Table II shows the amounts of the other preparations causing 75 per cent growth inhibition of the Singapore strain. Their potency exceeded that of preparation 17K only slightly.

Experiment 6. This experiment was performed to exclude the possible role of specific antibodies.

Table III

*Inhibition of viral multiplication in suspended chicken allantoic membrane fragments**

Inhibitor preparation	Virus added	Centrifugation	Heating	Inhibition per cent
Homologous serum 1 to 360	—	—	—	75
Homologous serum 1 to 20	—	—	95° C	0
Prep. No. 8. 2.4 HAIU	—	—	—	75
	—	—	95° C	70
	—	20 000 g 1 hour***	—	78
	1024 HAU**	—	95° C	75
Prep. No. 8. 24 HAIU	1024 HAU**	20 000 g 1 hour***	95° C	0
	1024 HAU**	20 000 g 1 hour***	95° C	0
Prep. No. 8. 24 HAIU	1024 HAU**	20 000 g 1 hour***	95° C	0

* Inoculum, 10 000 ID₅₀ of Budapest 4/49 in each case

** Budapest 4/49. Kept at room temperature for 48 hours after addition to the inhibitor

*** supernatant tested

Table III shows that anti-Budapest 4/49 serum diluted 1 : 360 caused 75 per cent inhibition in the multiplication of the homologous strain in HORVÁTH's roller drum. To obtain data on the resistance of antibody to the damaging effects to which the Francis inhibitor was subjected during preparation, one part of the same serum was diluted in 9 parts of a pH 7.2 buffer and kept in a boiling water bath for 30 minutes. After removing by centrifugation the fine precipitate which had formed, the supernatant was diluted with an equal volume of HORVÁTH's glucosol medium [11], and tested for inhibitory activity against the homologous strain. No inhibition could be observed whereas the modified Francis inhibitor, if treated similarly, retained its full activity.

Experiment 7. To obtain some information on the mode of inhibition exhibited by the modified inhibitors, Budapest 4/49 virus was added to an equal volume of Preparation 8, and the mixture was kept at room temperature for 48 hours. In order to inactivate the virus whether free or bound but capable of interference, the inhibitor-virus mixture was heated to 95° C. After cooling the preparation possessed its full inhibitory activity.

The activity of the unheated inhibitor-virus mixture could not be exactly determined because of the presence of active virus which had been added in excess. It was therefore to be decided whether the inhibitor had been bound to the virus before heating or the inhibition is based on a mechanism entirely different from that supposed by us.

To elucidate this question, a mixture of active virus and Preparation No 8 after being kept at room temperature for 48 hours was centrifuged with

20 000 g for 1 hour. The precipitate, which was darker than the starting material, was discarded, and the supernatant, also coloured, was heated as described above, and then tested for its virus-inhibiting activity. It was found inactive, a proof that Preparation No 8 had sedimented with the influenza virus. It was shown, on the other hand, that Preparation No 8 alone did not settle under the same circumstances. It was therefore concluded that (i) the modified inhibitor was bound to the virus, and (ii) heating had liberated fully active inhibitor from this bond.

Inhibition in the embryonated egg and in the mouse lung. Experiment 8. Sixty 11 day old embryonated eggs were inoculated each into the allantoic cavity with 100 000 ID₅₀ of the Budapest 4/49 virus in a volume of 0.1 ml. Twenty of them (Group 1) were given 48 HAIU of Preparation No 8 in a volume of 0.2 ml 30 minutes before, another 20 eggs (Group 2) the same dose 30 minutes after, infection. At the latter point of time the adsorption of the virus had practically been over. The control group was treated only with the buffer before being infected. Table IV shows the distribution of the HA titres of the allantoic fluids harvested after 48 hours, and the average titres.

Table IV
*Inhibition of viral multiplication in the embryonated egg**

Group No.	Inhibitor	Time of adding inhibitor	HA titre**										To- tal	Geometric mean
			16	32	64	128	256	512	1024	2048	4096			
1	48 HAIU Prep. No. 8	30 min. before virus	2	1	2	5	4	4	1	—	—	19	1 : 154	
2	48 HAIU Prep. No. 8	30 min. after virus	—	2	5	1	—	3	4	3	1	19	1 : 330	
3	—	—	—	—	—	1	2	2	2	5	4	16	1 : 1217	

* Distribution by HA titre of individual allantoic fluids harvested from eggs infected with 10⁵ ID₅₀ of the Budapest 4/49 strain

** Reciprocals

Inhibition amounted to 87 per cent in group 1 and 73 per cent in group 2. The infective titres of the pooled allantoic fluids were also established (Table V) and submitted to the probit analysis.

If the geometric mean of infective titres for the control group is taken for 100, that for group 1 was 1.9 (fiducial limits, 0.8 and 4.3) and for group 2 2.7 (fiducial limits, 0.9 and 6.7). Thus, there was no significant difference between the groups treated with inhibitor at different times.

A similar experiment was performed with the Lee strain. The results were essentially the same.

Table V

Infectivity titration of the allantoic fluids from Experiment 8

Group No.	Dilution of allantoic fluids	HA positive cultures per cultures inoculated	log ID 50/ml
1*	10^{-7}	19/24	7.4
	10^{-8}	2/24	
	10^{-9}	0/16	
2*	10^{-7}	19/24	7.5
	10^{-8}	5/24	
	10^{-9}	0/16	
3*	10^{-7}	16/16	9.0
	10^{-8}	15/16	
	10^{-9}	10/16	

* See Table IV

Experiment 9. Two preliminary experiments were carried out concerning the inhibition of viral multiplication in the mouse lung. In both experiments each mouse was given intranasally 100 mouse-lung-ID50 of the mouse-adapted Lee strain. In *Experiment 9/a* each mouse received intranasally 6.4 HAIU of Preparation No 8 immediately after infection and 24 and 48 hours later. At 72 hours the infective titre of the pooled lungs was 2 log units lower than that of the control pool prepared from the lungs of mice receiving saline instead of inhibitor.

In *Experiment 9/b* each mouse received 6.4 HAIU of Preparation No 8 intravenously immediately after infection and further 32 HAIU every 12 hours intraperitoneally. The infective titre of the pooled lungs harvested at 24 hours was 10^{-2} whereas that in the appropriate control group was 10^{-4} . This difference disappeared by the 48th hour. These findings need further investigation.

Discussion

Mucopolysaccharides of various origin are known to inhibit influenza virus HA [12] competitively. Because of the lability of the virus-inhibitor complex viral multiplication is not inhibited by these substances [GREEN *et al.*, DE BURGH *et al.* 12, 14, 15]. In spite of this the idea of inhibiting viral multiplication by HA inhibitors is not new [16]. A HA inhibitor has been even found which has the capacity of inhibiting viral infection. The inhibitor, which is present in the intestinal tract of the mouse acts against the GDVII virus [17]. There exists, however, an essential difference between the latter type of inhibition and the inhibition of Myxoviruses by mucopolysaccharides, namely, that in the former case the virus-inhibitor complex is stable.

CURTAIN [10] coupled HA inhibitors of high molecular weight to powdered cellulose by a chemical method resembling that used by us. His coupled inhibitors were destroyed by influenza virus as readily as the dissolved one. The resistance of some of our coupled inhibitors to the viral action is not inconsistent with CURTAIN's finding since in our experiments, too, numerous aniline derivatives have failed to modify the effect of Francis inhibitor.

Most recently a HA inhibitor has been prepared [21] which inhibited the neurotoxic effect of the influenza virus in mice and the multiplication of the same virus in tissue cultures. The inhibitor, an ox brain ganglioside contains a neuramic acid-glucosamine component. There are no data available as to the stability of the ganglioside-virus complex.

The basic mechanism of the inhibition exerted by the compounds discussed in the present report is unknown. So is (i) the role played by the cell receptor in viral multiplication [2], and (ii) the enzymic activity of the influenza virus against cell receptors and mucopolysaccharide inhibitors [13, 22]. Still, the modified inhibitors appear to be suitable for investigating the first question, but owing to their antigenic activity [18] it is difficult to study their effect in animals. To eliminate the difficulties, readily absorbable, non-antigenic "modified" inhibitors are to be prepared. For practical purposes similar studies with the aniline derivatives of low-molecular inhibitors might be useful [19].

Depending on the coupled aniline derivative, some preparations inhibited viral multiplication while others failed to do so. It is thought that multiplication is inhibited by those compounds only in which a rearrangement of the molecular structure has made the neuramic acid ester linkage strong enough to resist to the virus without hindering the formation of virus-inhibitor complexes.

The mechanism of inhibition in the embryonated egg is even more obscure. Preparation No 8 proved to be inhibitory even if injected at a time when viral adsorption to the allantoic cells had been completed. It seems possible that an "inhibitor adsorption", similar to haemadsorption [23] interfered with the virus.

Investigation into the chemical nature of the modified inhibitors is in progress.

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Address of the author:

IVÁN HOLLÓS

State Institute of Hygiene, Gyáli út 2—6, Budapest IX, Hungary.

REGIONAL DISTRIBUTION AND CHARACTERISTICS OF VIRAL HEPATITIS IN HUNGARY DURING THE PERIOD 1952 TO 1957

By

KATALIN SOLT and I. VEDRES

State Institute of Hygiene, Budapest, and Institute for Public Health, University Medical School, Budapest

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Summary. The increase of viral hepatitis morbidity during the period 1952 to 1957 was not uniform for the different areas of Hungary. An influence of naturally acquired immunity on the morbidity could not be observed in the country, while it might be claimed to have appeared in the greater towns.

Morbidity, mortality and lethality were the highest in the Transdanubian area and the lowest in the Great Plain in the year 1957. The increase in morbidity in the settlements with a low number of inhabitants suggests a centrifugal character of spreading. In 1954 to 1957 the morbidity for both viral hepatitis and typhoid fever was higher in towns provided with a water and sewer system than in those without them. This suggests the importance of person to person transfer of the above enteral diseases.

There was no correlation between the number of physicians per district, the density of population, the ratio of home treated and hospitalized patients on the one hand and the morbidity rate on the other.

Since 1950, viral hepatitis has been compulsory to report in Hungary. As mentioned in a previous paper [1], it took 1 or 2 years until the physicians became used to reporting it. This is the reason for presenting in this paper only the data collected in the period from 1952 to 1957.

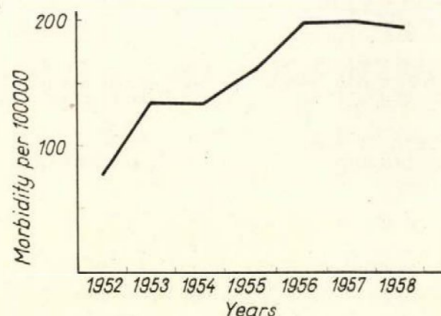


Fig. 1. Viral hepatitis morbidities in Hungary for the years 1952 to 1958 (per 100 thousand inhabitants)

The incidence of reported viral hepatitis cases exhibited a gradual increase from 1952 to 1956 (*Fig. 1*), while since that time it has remained unchanged. This increase in incidence during the period examined was not uniform for the different regions of the country.

In 1952, very probably because of failures in reporting, a morbidity over 101 per 100 thousand was reported only from the towns Budapest, Debrecen, Pécs and Szeged and from Komárom county. The lowest morbidity — less than 25 per 100 thousand — was reported from Tolna county (*Fig. 2*). The discrepancy was even more marked in 1955 (*Fig. 3*), when it was 97.3 per 100 thousand in Hajdú-Bihar county while 215.7 in Komárom

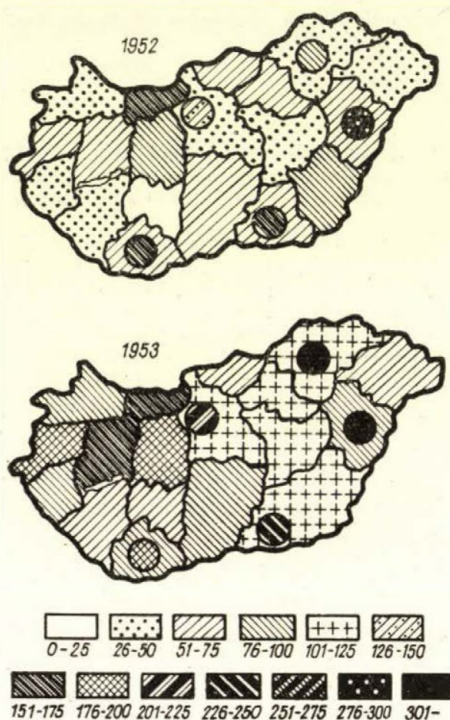


Fig. 2. Viral hepatitis morbidity in Hungary (per 100 thousand inhabitants)

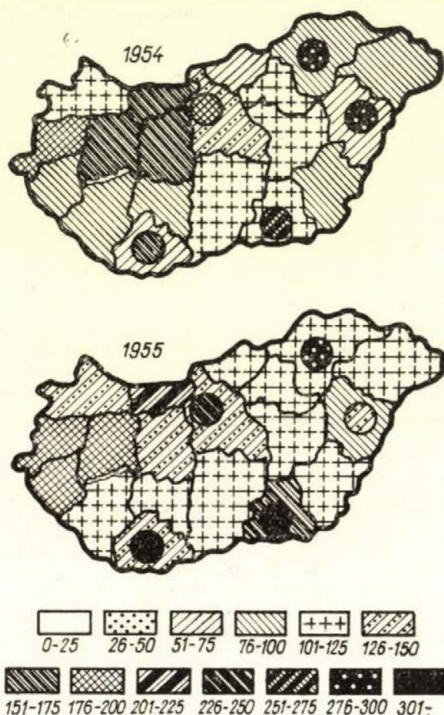


Fig. 3. Viral hepatitis morbidity in Hungary (per 100 thousand inhabitants)

county, and it only increased in the years 1956 and 1957 (*Fig. 4*), when it became observable also in Transdanubia and the neighbouring north and eastern counties Nógrád and Pest. The increase in morbidity was characteristic of the whole of Transdanubia except county Győr, where the lowest morbidity rate (101.2 per 100 thousand) was actually observed in 1957.

During the period analysed the morbidity was remarkably higher in Budapest and the greater towns than in their surroundings and the average morbidity for the whole country. RAŠKA [2] observing a similar phenomenon suggested the presence of sufficient number of susceptible persons in the greater towns as a reason for the continuity of the epidemic process. No effects

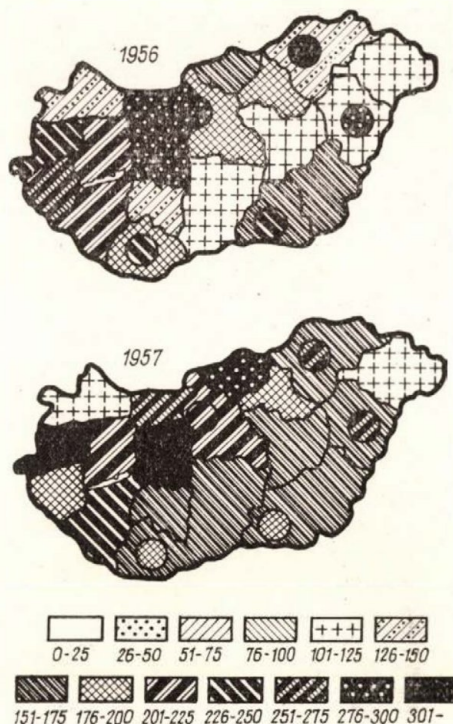


Fig. 4. Viral hepatitis morbidity in Hungary (per 100 thousand inhabitants)

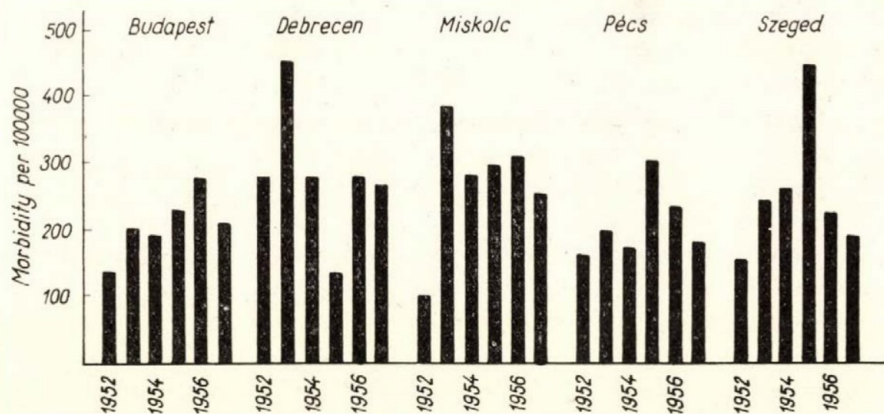


Fig. 5. Viral hepatitis morbidity in the great towns in 1952 to 1957 (per 100 thousand inhabitants)

of naturally acquired immunity could be observed in the morbidity curves for the different counties in the period of 1952 to 1957. The possibility of such an effect, however, could not definitely be excluded for the greater towns (Fig. 5). These signs were more suggestive for the towns Debrecen, Szeged and Pécs than for Miskolc or Budapest.

Table I
Viral hepatitis morbidity and lethality in different areas in 1957

Area	Morbidity	Mortality	Lethality
Great Plain	166.0	0.9	0.5
Northern Mountains	200.1	1.3	0.6
Transdanubia	219.0	1.7	0.7
Total (except Budapest)	191.5	1.2	0.6
Budapest	210.9	2.1	1.0
Total for the country	194.9	1.4	0.7

The morbidity, mortality and lethality for the different areas in 1957 were as follows (*Table I*). Morbidity was the highest in Transdanubia (west) and the lowest in the Great Plain (east). Lethality exhibited a similar distribution except in Budapest. The reason for this is unknown and a complex clinical-epidemiological study could only yield further information. Nevertheless, when evaluating the lethality figures for Transdanubia and the Great Plain, the differences in morbidity in these two areas largely suggest the influence of factors other than a mere failure in reporting.

No correlation was observed between the number of viral hepatitis cases reported and the ratio of inhabitants per one district physician. It was not possible, however, to obtain information about the frequency of consulting the physician in the different areas, thus we lack data of certain importance. As to the possible correlation between the density of population and the number of reported epidemic hepatitis cases in counties and greater towns, nothing of significance could be demonstrated.

Apparently, a detailed explanation of the differences in morbidity within the different regions could be obtained only by a thorough systematic local analysis of several factors, such as facilities for early diagnosis and early hospitalization, the nature of preventive measures applied, the life and civilization standards of the local population, the chances of contact infection during work, housing and other circumstances, etc. Nevertheless, the importance of appropriate reporting must not be disregarded, particularly as the reported morbidity rate for dysentery was also the lowest in the counties Győr-Sopron and Szabolcs-Szatmár, in the period from 1951 to 1955, as already reported [3].

There was no correlation between the number of hospitalized patients and the morbidity for viral hepatitis. This might have been due to the fact that most of the cases were diagnosed and the patient was sent to hospital only after jaundice had appeared, and during the long prodromal phase there were ample possibilities for the propagation of the infection [4, 5]. This could be avoided only by methods facilitating early diagnosis. Hospitalization of

the hepatitis patients was still inadequate in 1957, amounting to an average of only 76.3 per cent for the whole country. The lowest hospitalization rates were found in the industrial counties *e.g.* in county Borsod, 52.0 per cent, in county Baranya, 58.0 per cent; in county Komárom, 61.6 per cent.

During the period examined the morbidity for the settlements was found to vary according to the number of inhabitants. This observation is presented in *Fig. 6* where the villages and towns have been divided into eight categories according to their population, while Budapest is presented separately.

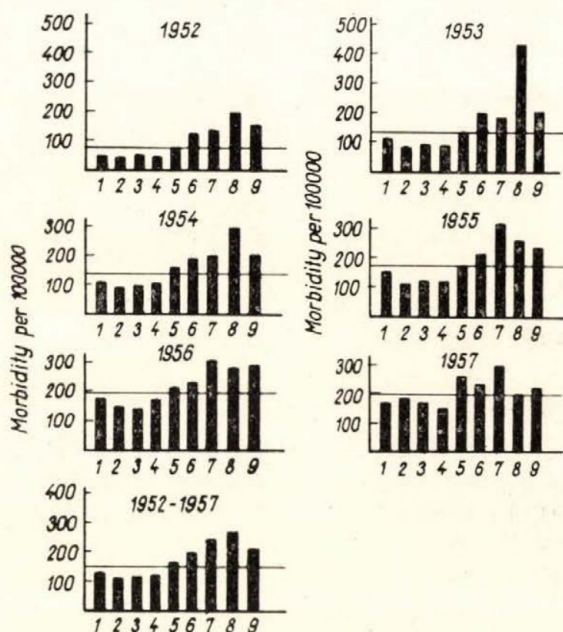


Fig. 6. Reported cases of viral hepatitis per 100 thousand inhabitants as related to settlements with different numbers of inhabitants (1952–1957)

- | | |
|------------------------|-------------------------------------|
| 1. — 1 000 inhabitants | 6. 20 001—50 000 inhabitants |
| 2. 1 001—3 000 | 7. 50 001—100 000 |
| 3. 3 001—5 000 | 8. 100 001—150 000 |
| 4. 5 001—10 000 | 9. Budapest |
| 5. 10 001—20 000 | — Average morbidity for the country |

When examining the different categories, a stepwise increase of morbidity seems to have occurred from 1952 to 1957. A decrease was observed only in the year 1957 in towns with a population over 100 thousand. The average morbidity for the whole country was essentially similar in 1956 and 1957. This means that while morbidity decreased in the greater towns, it increased in the smaller ones.

When examining the average viral hepatitis morbidity for the village categories up to 10 thousand inhabitants, there were no significant differences

in the period from 1952 to 1957. On the contrary, in categories 5 to 8 (see *Fig. 6*) a gradual increase occurred during the same period. Towns with 100 thousand to 150 thousand inhabitants exhibited the highest morbidity in the period between 1950 and 1954. From 1955 to 1957 this increase shifted to the lower categories, *i. e.* to towns with 50 to 100 thousand inhabitants, where morbidity was generally higher than, or equal to that, observed for Budapest.

In 1952 and 1953 the highest morbidity exceeded 4.8 to 5.3 times the lowest figures. This difference decreased gradually to 2.1 and 1.9 times in 1956 and 1957, respectively. This nivellation in the hepatitis morbidity of localities of different numbers of inhabitants appeared to be the result of a number of factors. The well-known centrifugal character of spreading of the disease [6] may have been one of them. The increasing tendency of even moderately ill patients to consult a physician, a result of the increasing standard of life and civilization, the improved knowledge of country physicians about the epidemiology and diagnosis of viral hepatitis, improvements in reporting and some other factors must all have contributed to it. As the spread of hepatitis by inoculation is generally known, one should not disregard the role of extensively used injection therapies, the increased application of treatments by means of not always correctly sterilized instruments, and freely performed transfusions.

Table II

Percentage of subjects 0 to 14 years of age among hepatitis patients in 1957

Areas with the lowest morbidity		Areas with the highest morbidity	
County Győr	47.3	County Vas	66.2
County Szabolcs	49.0	County Fejér	60.1
Town Pécs	35.5	Town Debrecen	55.0

In 1957 the age distribution was also examined both for the counties and the towns exhibiting the highest and the lowest morbidity rates. As there were no exact data available on the actual age distribution of the population we could only estimate distribution ratios instead of age-specific morbidity (*Table II*). The ratio of hepatitis patients between 0 and 14 years of age was higher in the areas with high morbidity than in those with a low morbidity rate. The average incidence of hepatitis patients between 0 and 14 years of age was 51.2 per cent for the whole country in 1957. No definite conclusions can be drawn from these figures; we only mention that according to literary data in endemic areas an overwhelming majority of the hepatitis patients is in the child age [7].

The seasonal morbidity curves for the different areas did not differ from that for the whole country [1], though in seven counties it very much resembled

that for Budapest, *i.e.* after a September, October and November peak the morbidity decreased. For the towns Miskolc and Szeged a late summer and early autumn increase characteristic also of other enteric diseases was observed, with the one month delay corresponding to the long incubation of hepatitis.

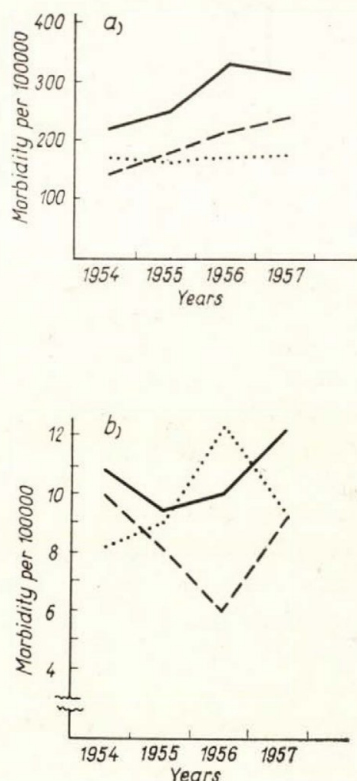


Fig. 7

- a) Viral hepatitis morbidity in towns with 20 thousand to 100 thousand inhabitants in 1954 to 1957 (per 100 thousand inhabitants)
 b) Typhoid fever morbidity in towns with 20 thousand to 100 thousand inhabitants in 1954 to 1957 (per 100 thousand inhabitants)

— Drained
 Storm-water drainage
 — — — Undrained

According to literary data there is a correlation between viral hepatitis morbidity and sewage [4, 8]. GELPERIN [9] found a 6.1 times higher incidence of hepatitis among people living in houses without sewers as compared to those houses provided with drain-pipes. According to ROYTMAN's survey [10], only 26.6 per cent of the hepatitis patients were living in houses with sewers. We studied the morbidity of viral hepatitis in the period between 1954 to 1957 in towns of 20 to 100 thousand inhabitants from the point of view of

Table III
Typhoid fever and hepatitis morbidity in 1954 to 1957 in towns with 20 to 100 thousand inhabitants

Designation	Viral hepatitis morbidity* Years				Average
	1954	1955	1956	1957	
Sewer system	219.6	244.2	339.2	315.5	280.6
Storm-water drainage only	171.7	161.4	173.7	175.6	170.6
Undrained	139.4	178.2	214.6	231.9	191.5
Typhoid fever morbidity*					
Sewer system	10.8	9.4	10.0	12.2	10.6
Storm-water drainage only	8.1	9.0	12.3	9.3	9.1
Undrained	10.0	8.1	6.0	9.1	7.9

* Calculated for 100 thousand inhabitants

general sewer-system, storm-water drainage and no drainage at all (*Table III*). Morbidity was higher for the towns with a sewer-system than for those lacking it. As this was in contradiction to the literary data it seemed necessary to perform similar investigations concerning another enteral infection of well-known epidemiology. Typhoid fever was chosen for this purpose. The results obtained resembled those of epidemic hepatitis (*Fig. 7*). It should be mentioned, however, that there is a great variety in the extent of drainage even in the towns with a sewer system. The extent of general drainage covers 30 to 95 per cent of the living accommodations in different towns, while the same figures are 3 to 10 per cent for towns provided only with storm-water drainage. From the data available it was not possible to establish an eventual relation between extent of drainage and incidence of viral hepatitis in the corresponding districts of the towns examined. Thus our results suggest the person to person transfer to be the most important factor in spreading viral hepatitis, similarly to other enteral infections. Even therefore the importance of virus carriers and latent infections must not be disregarded.

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Address of the authors:

KATALIN SOLT, State Institute of Hygiene, Gyáli út 2—6, Budapest IX, Hungary.

ISTVÁN VEDRES, Institute for Public Health, University Medical School, Mária u. 40, Budapest VIII, Hungary.

STUDIES ON THE LIPASE-SYSTEM OF LEPTOSPIRAE

I. TRIBUTYRINASE ACTIVITY

By

[L. BERTÓK and F. KEMENES

*Institute of Epizootiology, School of Veterinary Medicine, Budapest, and
Department of Animal Physiology, Animal Breeding Research Institute, Budapest*

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Summary. The lipase production by pathogenic and saprophytic *Leptospirae* has been studied by COMFORT's method. Strains capable of splitting animal fats were hydrolysing natural oils and tributyrin as well. However, the decomposition of animal fats by saprophytic *Leptospirae* was less intensive, while tributyrin was markedly hydrolysed by these organisms. The investigations provided data as to the appearance of leptospiral lipase (tributyrylase) in the culture-fluids and its relationship to haemotoxin. Inactivation by heat, filtration and dialysation of leptospiral lipase were also studied.

A comparison between the tributyrinase activity of pathogenic and saprophytic *Leptospirae* of our culture collection has revealed a considerable difference in the lipase systems of the two kinds of organism. The rate of leptospiral lipase reaction and its inhibitions by sodium arsanilate, neoarsphenamine and sodium fluoride have also been examined.

As has been concluded, investigations into the biological properties of *Leptospirae* would supply more information about the pathogenesis of leptospiroses, the distribution of serotypes in a certain area and other field-observations concerning the disease than detailed studies of the antigenic structure of these pathogenic agents.

Lipase production by pathogenic *Leptospirae* has been clearly demonstrated by the slide method [8] in KORTHOFF's cultures. This method, however, is not suitable for quantitative purposes, or even for precise qualitative evaluation, as the decomposition of different fats appears to run a similar way on the effect of swine pancreatic lipase, in fat-splitting bacterial (freshly isolated pseudomonad and staphylococcal) and leptospiral cultures. Neither does the method suit itself for studying the decomposition of oils and synthetic triglycerides containing a small number of carbon atoms.

Materials and methods

Pathogenic *Leptospirae* [5, 8] and saprophytic *L. biflexa* strains [4, 9] were used from the collection of this laboratory. Cell counts of 10 to 14-days KORTHOFF's cultures were made under a dark-field microscope, then the suspensions were standardized to contain 2.10^8 to 8.10^8 *Leptospirae* per ml.

For assaying the lipase activity of *Leptospirae*, COMFORT's method [3] was somewhat modified. Preliminary investigations showed that when the substrate was applied in a standard quantity, from 1 up to 8 ml of enzyme solution, the correlation between enzyme concentration and activity was linear. For the sake of economy, in these trials 1 ml standardized culture (enzyme solution) samples were used.

Tributyryl served as the substrate mixed with an equal part of 5 per cent acacia gum solution to obtain an emulsion. As a preservation, 0.2 per cent sodium benzoate was added. Before use the emulsion was diluted with a pH 7 phosphate buffer.

In preliminary experiments the optimum tributyrin concentration was found between 0.2 and 0.3 M (0.2438 M corresponded to 1 ml tributyrin, i. e. to 2 ml acacia gum emulsion

at 20° C and 1.0324 sp. gr.). Above this value enzyme activity, which was plotted against the molar concentration of tributyrin, scarcely showed a rising trend.

Apart from tributyrin, olive, sunflower, sesame, castor and linseed oils were used as substrates. The emulsification of these oils was carried out like that of tributyrin.

Measurement of lipase activity. Into 50 ml Erlenmeyer flasks 8 ml pH 7.0 buffer solution and 3 ml distilled water were pipetted. Then 1 ml KORTHOFF's culture was washed in the mixture and 2 ml tributyrin emulsion was added. Blanks were prepared in each trial by inactivating the enzyme prior to the addition of tributyrin for 20 minutes in a 100° C water bath. Then the flasks were closed with rubber stoppers and incubated for 24 hours at 37° C. Finally to each flask 3 ml 96 per cent ethanol and phenolphthalein indicator were added and the digestion mixture was titrated with *N*/20 NaOH until a pink colour appeared.

Considering that KORTHOFF's medium contained 10 per cent rabbit serum which had itself some lipase activity even after subsequent inactivations at 56° C, before use the activity of every new series of inoculated media was measured. The results of the experiments were corrected according to the activity exhibited by the uninoculated media. Lipase activity was calculated by subtracting the number of ml of *N*/20 NaOH used for the blank plus the uninoculated medium from the number of ml of NaOH used for the test cultures. This value was multiplied by the factor of the NaOH solution.

Calculation was performed as follows. One ml *N*/20 NaOH is equivalent to 0.0044 g butyric acid, i.e. to 0.00504 g hydrolysed tributyrin. Accordingly, to express the enzymatically decomposed tributyrin in grams, the corrected quantity of NaOH in millilitres should be multiplied by 0.00504. This number (*x*) being very small, for presentation in the Figures it has been multiplied by thousand.

When inhibitors were applied, 1 or 2 ml distilled water was substituted in the reaction mixture with an adequate concentration of the substances mentioned. The end concentration of these was for sodium arsinitate and neoarsphenamine 0.000071, for cysteine HCl 0.00071, for sodium fluoride 0.0071 *M*/1.

The accuracy of the method was evaluated statistically. The standard deviation for 100 examinations was $\sigma = \pm 0.106$ ml, i.e. 0.0005 g hydrolysed tributyrin (Avg. dev. = $\pm 1.81\%$).

Results

As shown by *Figs. 1* and 2, lipase (tributyrynase) production in the cultures of lipolytic *Leptospirae* increased proportionally to the growth of the organisms. It reached the highest level during the second week of cultivation when the peak of the growth curve nearly coincided with the curve of lipase activity.

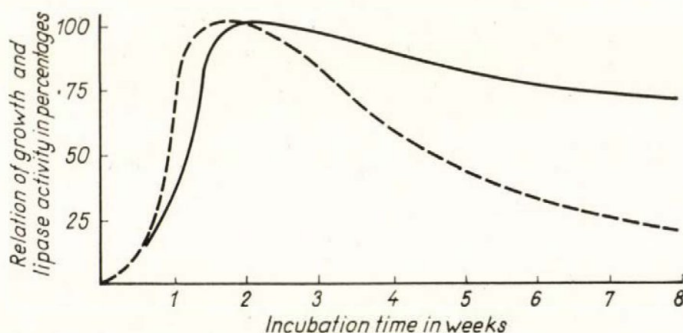


Fig. 1. Lipase (tributyrynase) production of virulent *L. icterohaemorrhagiae* (strain RS). Substrate: 0.2438 *M* tributyrin. Maximum cell count: 6.0×10^8 /ml, maximum tributyrinase activity: $x \cdot 10^3 = 22.15$, *x* = hydrolysed tributyrin in grams

————— Lipase activity
 - - - - - Growth

In *L. pomona* cultures lipase appeared earlier and persisted longer than haemotoxin (Fig. 2), and its activity decreased only slightly. Haemotoxin activity was independent of the viability of *Leptospirae* [6]. The same was the case with lipase which was often found to be present in several months old cultures from which no subcultures could be prepared. The independence of lipase

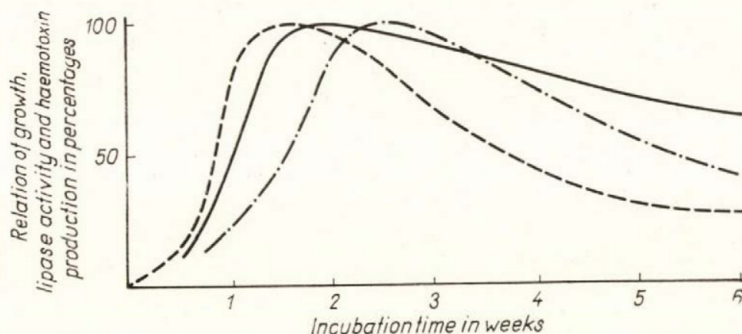


Fig. 2. Lipase (tributyrynase) activity, haemotoxin-production and growth curve of *L. pomona* (strain Nagymágócs)

— Lipase activity
 - · - · - Haemotoxin
 --- Growth

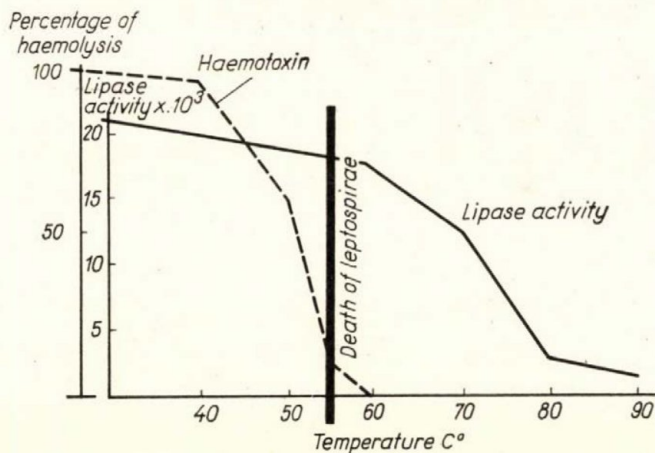


Fig. 3. Inactivation of *L. pomona* haemotoxin and lipase in 10 minutes at various temperatures. Substrate: 0.2438 *M* tributyrin

activity from viability was confirmed by heat-inactivation. As shown by Fig. 3, in contrast to haemotoxin, leptospiral lipase was comparatively thermo-resistant; its activity decreased just slightly at 56° C, a temperature killing the leptospirae.

Similarly to haemotoxin, leptospiral lipase could not be dialysed, but passed through the Seitz filtre. The lipase activity of such filtrates approx-

imately corresponded to that of the cultures, provided large quantities, at least 50 to 100 ml, of 2 or 3 week old cultures were filtered. From smaller volumes in contrast to haemotoxin, a considerable amount of lipase was adsorbed on the filtre.

As regards the substrate specificity of leptospiral lipase, in addition to tributyrin a number of natural oils (olive, sunflower, sesame, castor and linseed oil) were hydrolysed by KORTHOFF's cultures of pathogenic and saprophytic

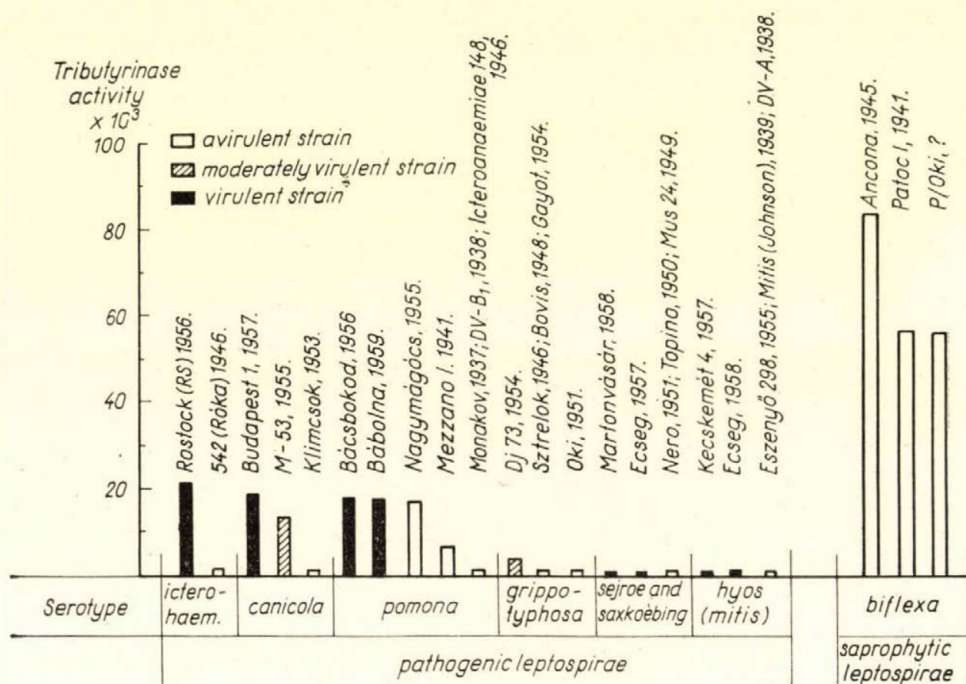


Fig. 4. Comparison of tributyrinase activity in various *Leptospira* strains

Leptospirae. Investigations with a different method are now in progress, to study the splitting of other lipoids, especially of pathogenetically important phospholipids.

As saprophytic *Leptospirae* maintained in the laboratory for long periods were still capable of splitting triglycerides [4], we studied the differences in lipase activity between the former organisms and the lipolytic, perhaps more invasive pathogens.

Fig. 4 illustrates an important difference concerning the tributyrinase activity of pathogenic and saprophytic *Leptospirae*. Tributyrinase from saprophytes splitted considerably more tributyrin than the pathogens did. Alternatively, animal fats (among others beef tallow) were decomposed much less

by the saprophytes than by certain pathogenic *Leptospirae*. The most striking evidence was offered by the different haemolytic titres of the fatty acids set free from the tallow (*Fig. 5*).

Thus the results demonstrated by the slide technique [8] in KORTHOFF's cultures were confirmed by the tributyrin and natural oil splitting ability of pathogenic *Leptospirae*. Only the virulent and serotypes (*L. icterohaemorrhagiae*, *L. canicola*, *L. pomona* etc.) of more invasive potency were markedly splitting triglycerides. On the other hand, the virulent Hungarian strains of serotypes belonging to *L. sejroe*, *L. saxkoebing* and *L. hyos* did not exhibit such activity.

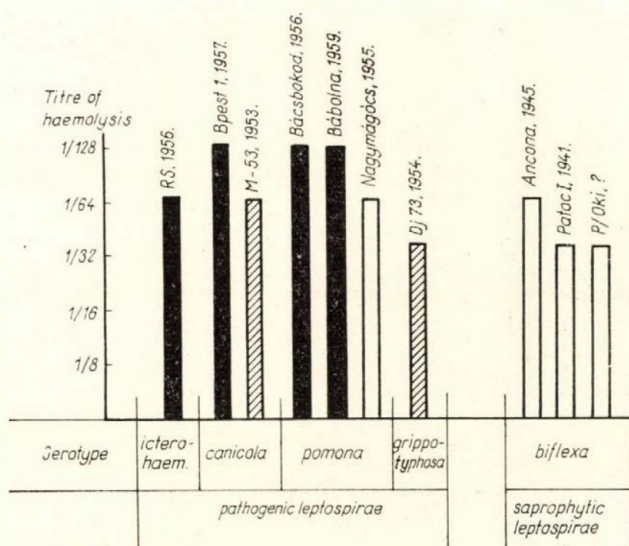


Fig. 5. Fatty acid haemolysis of sheep erythrocytes [8] by cultures of lipolytic *Leptospirae* decomposing beef tallow

In *Fig. 6* the heat-inactivation of lipase produced by saprophytic and pathogenic *Leptospirae* is presented. While the higher tributyrinase activity of saprophytic organisms decreased rapidly at 75° C, the lipase of the pathogens was inactivated only gradually.

It was also found that saprophytic *Leptospirae* which had been maintained in laboratories by culture transfer over 20 years [1] did not lose their lipase activity. In contrast to the pathogenic strains, which had originally been lipolytic organisms, such a long storage caused mostly the loss of virulence together with the loss of haemotoxin and lipase production.

The haemotoxin and lipase production cannot, of course, be associated with virulence. For example, the *L. pomona* (strain Nagyágócs) 1953 is an

active haemotoxin and lipase producer without being able to multiply in animals even after intraperitoneal injection [6]. When a mass culture is prepared from this organism and its germ-free filtrate is intravenously inoculated into young lambs and roe deer [4], the animals will inevitably die with haemoglobin-aemia, haemoglobinuria, jaundice, anaemia etc. This strain, similarly to the excellent toxin producer *Corynebacterium diphtheriae* Park No. 8 strain, has lost its ability to multiply within the living organism, which perhaps is an essential factor of virulence.

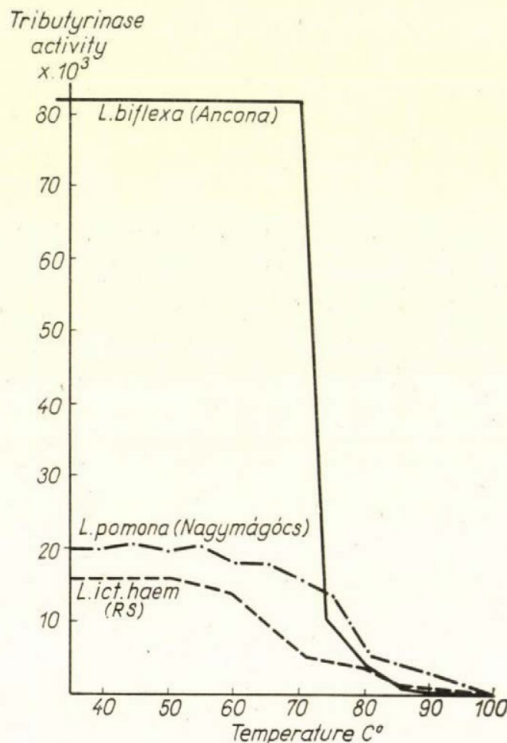


Fig. 6. Inactivation of tributyrinase produced by saprophytic and pathogenic *Leptospirae* in 10 minutes at various temperatures

When the activity of leptospiral lipase is plotted against the incubation time, or in other words when the rate of the reaction is represented, within a certain period (4 to 12 hours) the enzymic process can be regarded kinetically as of a zero reaction; provided that the substrate is added in excess (0.2438 *M* tributyrin). A zero reaction is characterized by the equation $K = x/t$. The value x/t being approximately constant, the points of the graph fall along a straight line (Fig. 7). Probably in consequence of the strong buffering capacity and to the excess of substrate, the equilibrium of the reaction was not

complete by the 64th hour; thus the activity plotted against time still had some rising tendency.

The rate of leptospiral lipase reaction could be altered by inhibitors. In these trials sodium arsenilate, neoarsphenamine and sodium fluoride were tested. As an activator or reactivator, cysteine HCl was used (Fig. 8). These investigations showed that sodium arsenilate in a concentration of 0.000071 *M* caused a 20 per cent decrease in the rate of the reaction. Neoarsphenamine in the same concentration had a similar effect, but on longer incubation, the results could not be evaluated owing to the quick breakdown (the solution

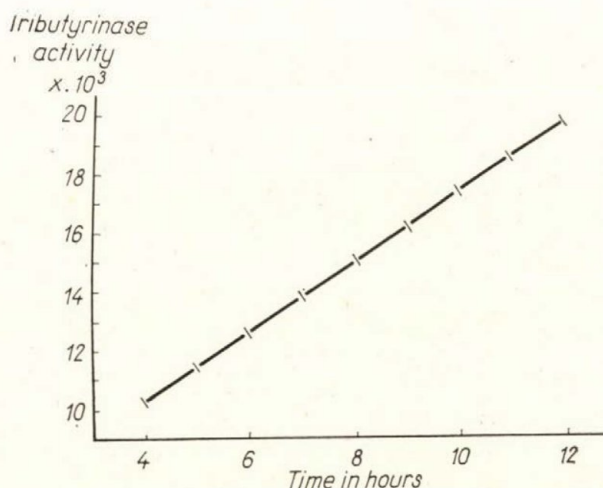


Fig. 7. Rate of tributyrinase reaction in *L. pomona* (strain Nagymágócs)

Substrate: 0.2438 *M* tributyrin

Order of the reaction: 0, $K = x/t$

became yellow). Inhibition by sodium arsenilate could be decreased to 6 per cent by the simultaneous addition of 0.00071 *M* cysteine HCl. Thus the rate of reaction in the latter system nearly reached that in the control with untreated enzyme.

The activity of leptospiral lipase is probably greatly dependent on the number of SH groups in the protein molecule of the enzyme. The arsenic in sodium arsenilate presumably exhibits its inhibitory action on these very SH groups, as this inhibition can be antagonized by the addition of a sufficient quantity of cysteine HCl. To elucidate whether in the case of leptospiral lipase (tributyrinase) a competitive antagonism exists between sodium arsenilate and cysteine HCl further investigations are required. The latter compound alone acts as an activator, since in a concentration of 0.00071 *M* it increased the rate of reaction by 4 per cent. On the contrary, sodium fluoride in a concentration of 0.0071 *M* decreased the rate of reaction by 62.5 per cent.

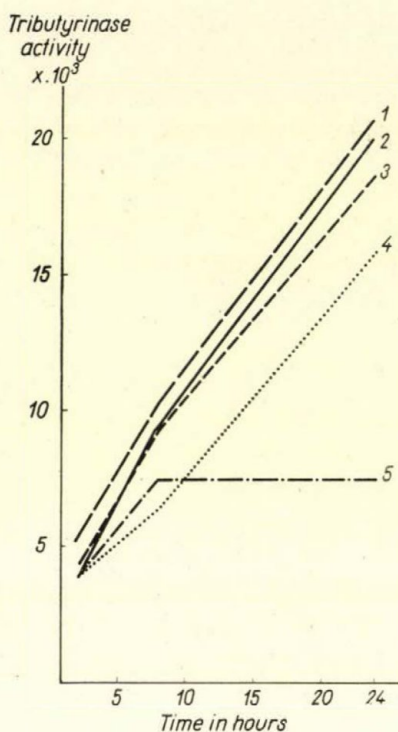


Fig. 8. The action of various inhibitors (activators) on the tributyrinase activity of *L. pomona* (strain Nagymágócs). Substrate: 0.2438 M tributyrin

1. Cysteine HCl (0.00071 M)
2. Untreated control
3. Sodium arsanilate (0.000071 M) + cysteine HCl (0.00071 M)
4. Sodium arsanilate (0.000071 M)
5. NaF (0.0071 M)

Discussion

The present trials have confirmed that lipase (tributyrinase) is produced by saprophytic and by certain pathogenic *Leptospira* strains. Among pathogenic *Leptospirae* those intensely splitting animal fats [8] are also able to hydrolyse natural oils and tributyrin. The saprophytes can also achieve this but they are splitting tributyrin even more intensively. However, the lipase system in saprophytic strains differs in several respects from that in invasive, more pathogenic *Leptospirae*. The results concerning the phospholipid decomposition will perhaps explain the fact that it is just the lipolytic pathogenic strains of more invasive potency (*L. icterohaemorrhagiae*, *pomona*, *canicola* etc.) which are principally spread all over the world. These observations might also explain why chiefly the latter serotypes are causing severe pathological lesions in guinea-pigs and Syrian hamsters (jaundice, haemorrhages in the lungs and

other organs). In our opinion the considerable differences in the lipase activity of virulent leptospirae belonging to various serotypes could shed more light on some field observations [7] than the detailed examination of antigenic structure especially if the former types of difference are assumed to occur within one and the same serotype. For example, *L. bataviae* prevalent in Java and Sumatra is as fatal to guinea-pigs as a virulent *L. icterohaemorrhagiae* strain. It may be imagined that the former has a more detrimental lipase activity than *L. bataviae* (*oryzeti*) occurring in *Micromys* mice of the North Italian rice fields. Such or similar biological differences might be responsible for the different course of the diseases caused by these serologically identical organisms. For example in Java and Sumatra human *L. bataviae* infection is properly called as "Indonesian Weil's disease" [10], while in Northern Italy the same serotype causes a mild infection among thousands of rice-workers without ever giving rise to jaundice or causing death [2]. Finally, investigations into the biological properties of pathogenic Leptospirae may throw more light on the pathogenesis of the disease, the simultaneous occurrence of various serotypes in a certain area and other field observations concerning leptospirosis than the detailed studies of the antigenic structure of these organisms.

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Address of the authors:

LÓRÁND BERTÓK, FERENC KEMENES,
Institute of Epizootiology, School of Veterinary Medicine, Hungária krt. 23, Budapest XIV, Hungary.

THE MICROBIOLOGY OF CURING

By

G. BIRÓ

Hungarian Meat Research Institute, Budapest

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Summary. The microbiological changes in brine were followed during a 20 day process of ham-curing. In the course of curing the total germ count decreased from 10^8 to 10^6 , with irregular fluctuations. *E. coli* could be demonstrated in the brine in the first 8 days only.

Two *Micrococcus* strains proved to be the strongest denitrifiers. Their denitrifying activity was found to increase in media of high salt content; beyond salt concentrations of 22 per cent they manifested a greater resistance to salt in the presence of saccharose. This phenomenon has been ascribed to the production of mucus.

The *E. coli* strains examined showed proliferation only in broth containing less than 13 per cent of salt. In the microbiological assessment of curing brine, the presence of *E. coli* is therefore indicative of inadequate handling, first of all of irregular dilution, of the curing brine.

Of the vast literature on the theoretical and practical problems presented by the microbiology of curing let us first mention LEISTNER's series of comprehensive studies on the bacterial processes of the curing solution, discussing in detail the germ content, the species of germs, the development of colour and aroma as well as their possible relation to decomposition of the brine, and the qualitative changes of the microflora of brines during the various phases of curing [1].

The *Second International Symposium on Food Microbiology* has dealt with numerous such questions as those of the culture media, conditions of cultivation, sampling, origin of the brine flora, survival of contaminating faecal bacteria etc [2, 3, 4, 5, 6]. There are data concerning the salt resistance of not only saprophytic microorganisms occurring on the surface of meat, but also of pathogens (*Salmonella*, *M. tuberculosis*) [9, 10, 11].

More or less successful attempts [8, 14, 15] have been made at practical utilization of the curing brine microflora, quick-curing with initiative bacterial cultures, improvement of colour and flavour.

As the process of curing advances, changes occur in the composition of the initial bacterial population under the influence of various factors. Some species of microbes are considerably reduced in number, others completely disappear, while again others, by gradual proliferation, develop into the dominant microflora of the brine. In number and importance alike, halophile Micrococci assume the foremost place in the ripe brine, but Lactobacilli, Pseudo-

monas, *Achromobacter*, *Proteus*, *Sarcina*, and many other species of bacteria are also encountered.

In the present study we shall deal with the denitrifying bacteria important for the development of colour, especially with the proportion of denitrifiers, the correlation of denitrification and salt concentration, the growth-promoting influence of sugar on bacteria in the curing brine, and the incidence and salt tolerance of *E. coli* in the solution.

In the selection of *Micrococcus* strains for his experiments aimed at improvement of colour, NIINIVAARA [8] considered strong denitrification and salt resistance in the first place.

As evidenced by data published in the literature, positive correlations may be traced between increased salt concentration and the degree of denitrification in a halophile flora [2, 3]. As regards the occurrence and salt tolerance of *E. coli*, different findings have been reported [4, 5, 6]. Thus, despite the existence of coli strains unable to multiply in the presence of 5 per cent salt, there are others that decompose nitrate even at salt concentrations of over 20 per cent. Such phenomena are known from the literature also in connection with *Salmonella* strains [9], and it is presumably unnecessary to stress the meaning of this circumstance from the point of view of the hygienic and microbiological evaluation of curing brines.

Materials and methods

In our investigations we followed the 20 day course of a ham curing process. Curing was performed at $+10^{\circ}$ C, in concrete vats. Samples were collected with sterilized pipettes at 30 cm depth after thorough stirring of the brine with a sterilized paddle. With the 200 ml material, taken every other day, cultivation was started in broth containing 3 per cent NaCl and 1 per cent of peptone, or in agar broth containing 1 per cent of peptone incubated at 20° C for 24 to 48 hours. The microflora was selected according to denitrification, and the most active strains were compared for salt resistance. NO_2 content was determined in 24 and 48 hour cultures in 5 ml peptone broth containing 1 per cent KNO_3 , 3 per cent NaCl, inoculated with a bacterial suspension in 1 ml physiological saline. Following the addition of two drops of GREISS—LOSVAJ's reagent to 1 ml of bacterial broth culture, the strains were differentiated on the basis of the colour reaction; then the same reagent was employed for more accurate comparison by photometry [12]. The NO_2 values obtained were expressed as per cent of the total NO_2 , computed by stoichiometry from the NO_3 content of the broth.

For studying the role of sugar, strains were grown in broth or agar with and without saccharose.

In order to facilitate the morphological examination of *Micrococci* forming a mucous material, bacterial suspensions were prepared with 1 per cent acetic acid which dissolved the mucus and rendered staining of the cocci possible.

The mucus derived from the *Micrococci* was assayed for carbohydrate content and analysed by paper chromatography [17, 18, 19] according to VAS and CSIBA [16].

After washing off the agar surface culture, the suspension was added drop-wise to a fourfold volume of 96 per cent alcohol containing 2 volume per cent of acetic acid, in order to precipitate the polysaccharide present in the mucus. After centrifugation the sediment was dissolved with 0.1 N NaOH and again subjected to precipitation with alcohol. The precipitate thus obtained was dried; a 4 per cent solution with $\text{N H}_2\text{SO}_4$, in sealed glass tubes was subjected to hydrolysis in a water-bath. After filling of the tubes, the hydrolysate was neutralized with BaCO_3 , a loopful of it was placed on a 3 cm wide Schleicher Schüll No 2043b filtrate paper strip and left to migrate for five hours in an acetone-butanol-water system

(7 : 2 : 1), and developed with aniline-diphenylamine (4 per cent alcoholic solution) and phosphoric acid (5 : 5 : 1).

E. coli strains grown on KLIMMER's or bromthymol blue-lactose-tripaflavine medium were cultivated in broth media of varying salt content; the rate of growth was assayed by turbidimetry [13].

Results

During the time of curing the total number of germs decreased with fluctuations from 10^8 to 10^6 . The plates made with different dilutions containing up to 200 colonies revealed wider variations in the proportion of denitrifier colonies to the total than were displayed by the fluctuations in the total number of germs. Until the middle of the period of curing, the proportion of denitrifiers

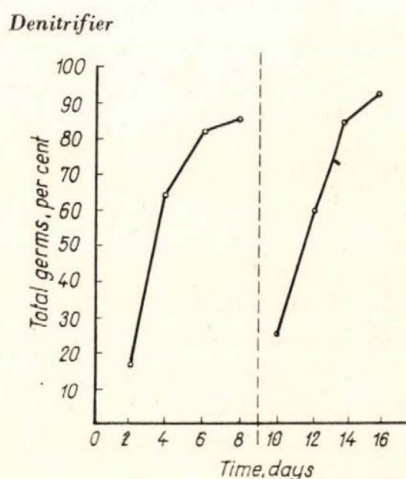


Fig. 1. Changes in the proportion of denitrifiers to the total bacterial count during the process of curing

rose from the initial 17 per cent to 87 per cent; after exchange of the brine and rearranging of the meat, the ratio of 26 per cent again increased to 93 per cent by the end of the process of curing (Fig. 1).

Thus in the course of the microfloral changes known from the literature (LEISTNER) the initially small proportion of denitrifiers increases during the period of curing until by the end the flora is composed almost exclusively of denitrifiers.

As regards denitrification, according to BERGEY's manual the Micrococci closely related to *M. denitrificans* and *halodenitrificans* are truly halophile organisms, whose proliferation and denitrifying activity improves on raising the salt concentration in the medium.

At high salt concentrations our Micrococcus strains M_1 and M_2 produced more nitrite from the culture medium, as shown in Fig. 2, which illustrates

an experiment where 5 ml peptone broth containing 1 per cent nitrate was inoculated with 1 ml of bacterial suspension (10^8 germs). Nitrate was determined in 2 ml culture by photometry.

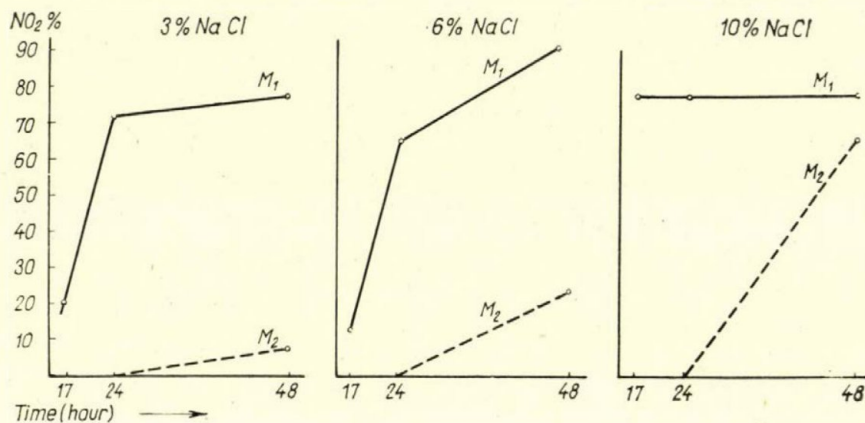


Fig. 2. Denitrification by strains M_1 and M_2 incubated at 20°C in culture media with varying salt content

Beside their known and supposed role played in curing brine [7], sugars undoubtedly offer an easily decomposed nourishment to bacteria. This does not yet account for the fact that under conditions varying as to sugar content but identical in other respects, Micrococci manifest a different behaviour regarding their resistance to salt. Strains that in the presence of 2 per cent saccharose thrive in 30 per cent salt, cease to grow in broth containing 26 per cent salt but no carbohydrate.

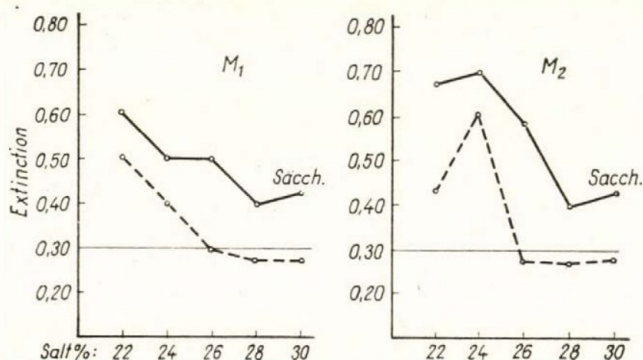


Fig. 3. Denitrification capacity of strains M_1 and M_2 in broths with varying salt content, with and without saccharose, incubated at 20°C for 48 hours.

○—○ medium with saccharose
 ○---○ medium without saccharose
 negative control: extinction, 0.30

Inoculation of culture media and determination of nitrate content, as in Fig. 2

The effect of sugars probably contributes to the development of the mucous capsule of Micrococci; this is supported by the following observations.

In cultures growing on media of high salt content it was noted that upon removal of the material with a loop, fine viscous threads could be drawn; at the same time the Micrococci did not satisfactorily take either Gram's or aniline dyes. The cocci, accumulated in large heaps and embedded in a mucous substance, remained inaccessible for the dye. There was no pronounced capsule formation, since the classical capsule stains (negative India ink, Johne's,

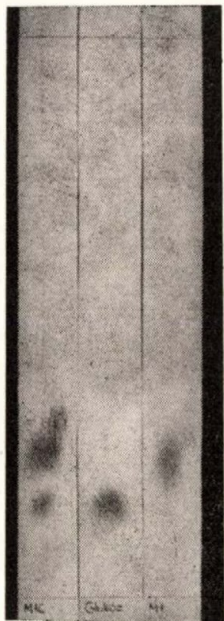


Fig. 4. M_1 : Chromatogram of hydrolysate of the mucous substance produced by strain M_1 grown on culture medium containing no saccharose.

Glucose: Chromatogram of glucose $M_1 C$: Chromatogram of hydrolysate of the mucous substance produced by strain M_1 grown on culture medium containing saccharose

Lupke's, Löffler's, etc.) failed to give a positive result. After incubation with 1 per cent acetic acid, however, the cocci already took Gram's stain.

Having ascertained the fact of enhanced salt resistance and mucus formation in culture media containing sugar, we proceeded to investigate the composition of the monosaccharide produced in media with and without 1 per cent saccharose. Without saccharose there appeared only one reduction spot (R_f , 0.23) on the chromatogram while from cultures containing saccharose several such points were revealed (R_f , 0.16; 0.26; 0.30). The R_f of one of these corresponded to the R_f characteristic of glucose (0.16) (Fig. 4).

The results of our investigations concerned with the demonstration of *E. coli* agreed with the findings of BUTTIAUX and MORIANEZ [5] in that the organism was present in the brine only in the first 8 days of curing. Two *E. coli* and two *A. aerogenes* strains of faecal origin were tested for salt resistance. The bacterial count established in broth with no extra salt (0.9 NaCl) incubated

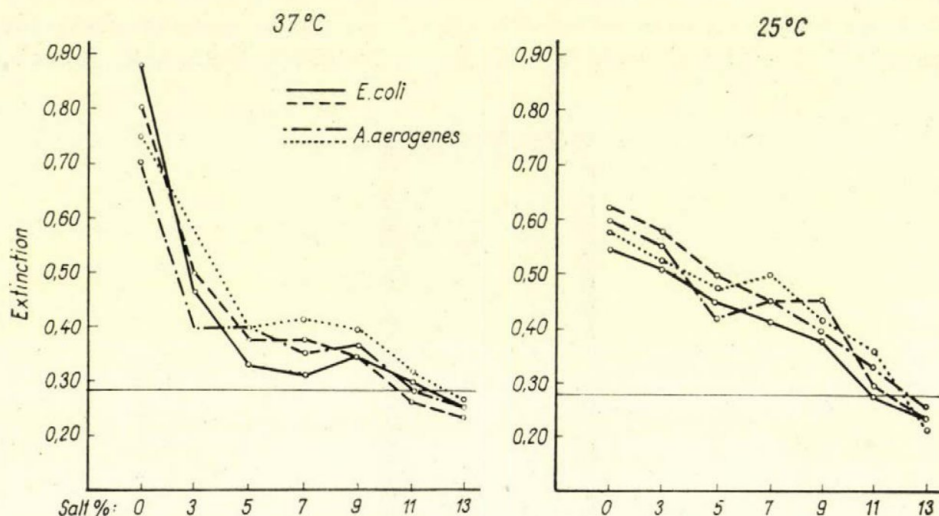


Fig. 5. Denitrifying activity of *E. coli* strains incubated for 48 hours in broths with varying salt content. 5 ml samples of broth were inoculated with 2 ml of a bacterial suspension (10^8 germs per ml)

at 37° C steeply diminished on the addition of 3 and 5 per cent salt; proliferation grew sparse at 7, 9 and 11 per cent; media containing 13 per cent salt yielded negative *E. coli* resp. *A. aerogenes* cultures (Fig. 5).

Acknowledgement. We are indebted to chief technician Miss I. TIMÁR for laboratory assistance.

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Address of the author:

GÉZA BIRÓ

Hungarian Meat Research Institute, Herman Ottó út 15, Budapest II, Hungary.

STUDIES OF A *CL. PERFRINGENS* PHAGE

II. THE LATENT PERIOD OF ITS GROWTH CURVE

By

GEORGINA GÁSPÁR

State Institute of Hygiene, Budapest

(Received January 19, 1960)

Summary. (i) One-step curve of the bacteriophage 808/3a of *Cl. perfringens* was established. The curve showed a slow rise during the "latent period".

(ii) The latent period was investigated by the method of "maturation inhibition". Maturation was inhibited by transferring samples from the growth mixture into aerobic growth tubes at different times.

(iii) Two stages of the latency can be distinguished: In the first stage newly-formed particles are not present; aeration at this time leads to a definite decline in the phage titre. In the second stage, from about the 35th minute of anaerobic incubation, new infective particles are present, and released even under aerobic conditions.

(iv) A possible explanation of the moderate rise during latency is that bubbling with N_2 gas disrupts the chains of *Cl. perfringens*, and so the phage particles originally adsorbed to the same chain are made capable of producing a separate plaque each.

(v) Aeration of either the *Cl. perfringens* culture or the phage preparation prior to preparing the adsorption mixture failed to cause any alteration in the latent period.

In a previous communication [1] we reported on some methods applicable in anaerobic phage research. A titration method, methods for preparation and standardization of antiphage sera, and for establishing the one-step curve were described. The growth curve of GUELIN's *Cl. perfringens* phage 808/3a was characterized, and the phage's heat sensitivity was examined. It was a peculiarity of the one-step growth curve established by using single-infected cells that during the "latent period" a continuous, moderate increase in the phage titre took place. The question arose whether this was a true latent period.

The latency of phage 808/3a is the subject of the present report. A method for stopping phage multiplication is described and discussed.

Materials and methods

Phage 808/3a and the sensitive *Cl. perfringens* strain were those used previously [1]. The phage was titrated and the one-step curve established as described in the same report. Multiplicity of infection was always < 1 . At the end of the adsorption time the bacterium-phage mixture (adsorption mixture) was highly diluted to make a "growth mixture". The last step of dilution was carried out by transferring the mixture into a growth tube containing previously boiled and cooled Hartley broth. The growth tube for anaerobic cultivation had a rubber stopper holding three tubes, one for taking samples, another for introducing N_2 gas, and a vent tube. In order to stop multiplication, air was introduced instead of N_2 .

Experimental

One-step growth curves. Fig. 1 shows five one-step curves from independent experiments. The average latency in the five experiments was 47 minutes, the average burst size 374. During the latency the phage count showed a slow but definite increase in each case.

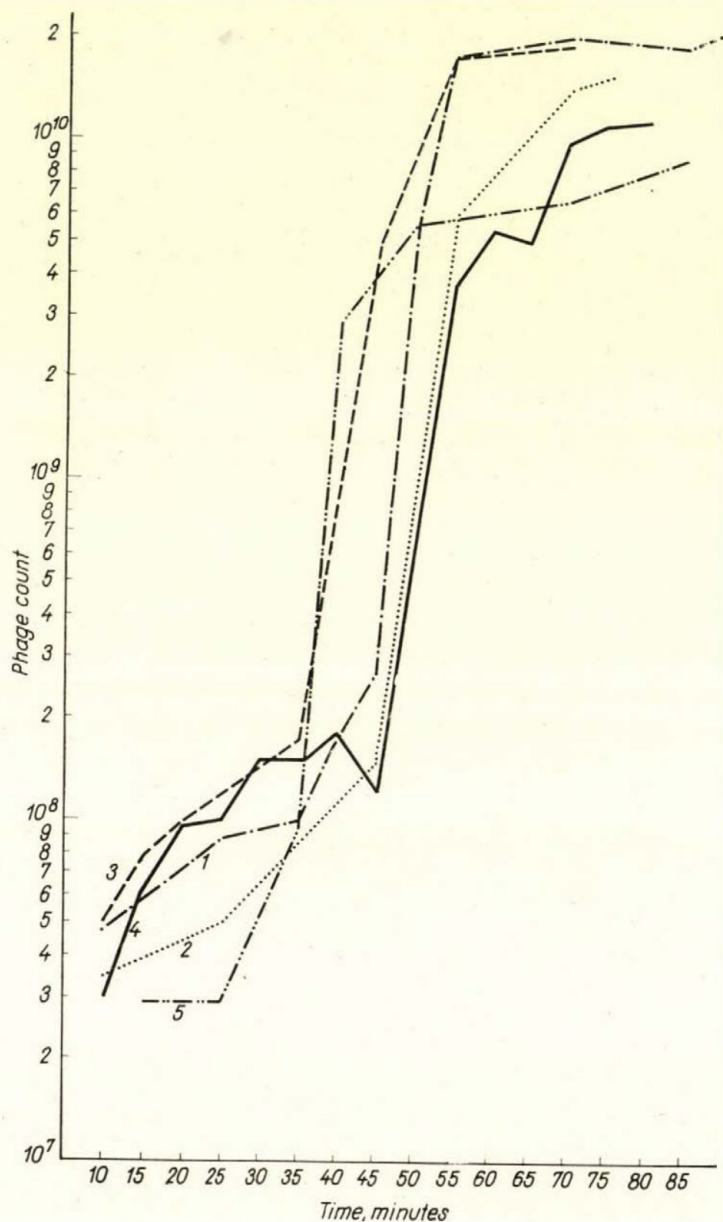


Fig. 1. Five one-step growth curves of *Cl. perfringens* phage

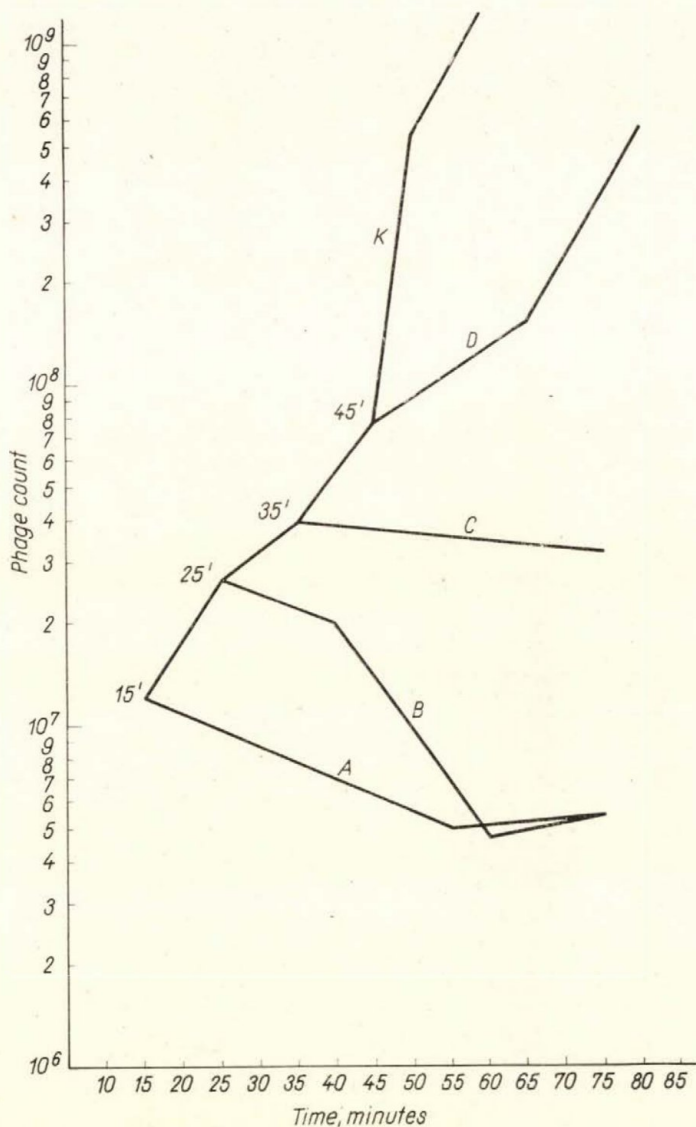


Fig. 2. Interruption during the latency of the growth curve of *Cl. perfringens* phage.

- A: interrupted at 15 minutes
- B: interrupted at 25 minutes
- C: interrupted at 35 minutes
- D: interrupted at 45 minutes
- K: control

Maturation inhibition. In order to characterize the latent period, we attempted to stop phage multiplication at different times.

The adsorption mixture was prepared and diluted as usual, and anaerobic incubation was initiated. From the growth mixtures samples were taken at

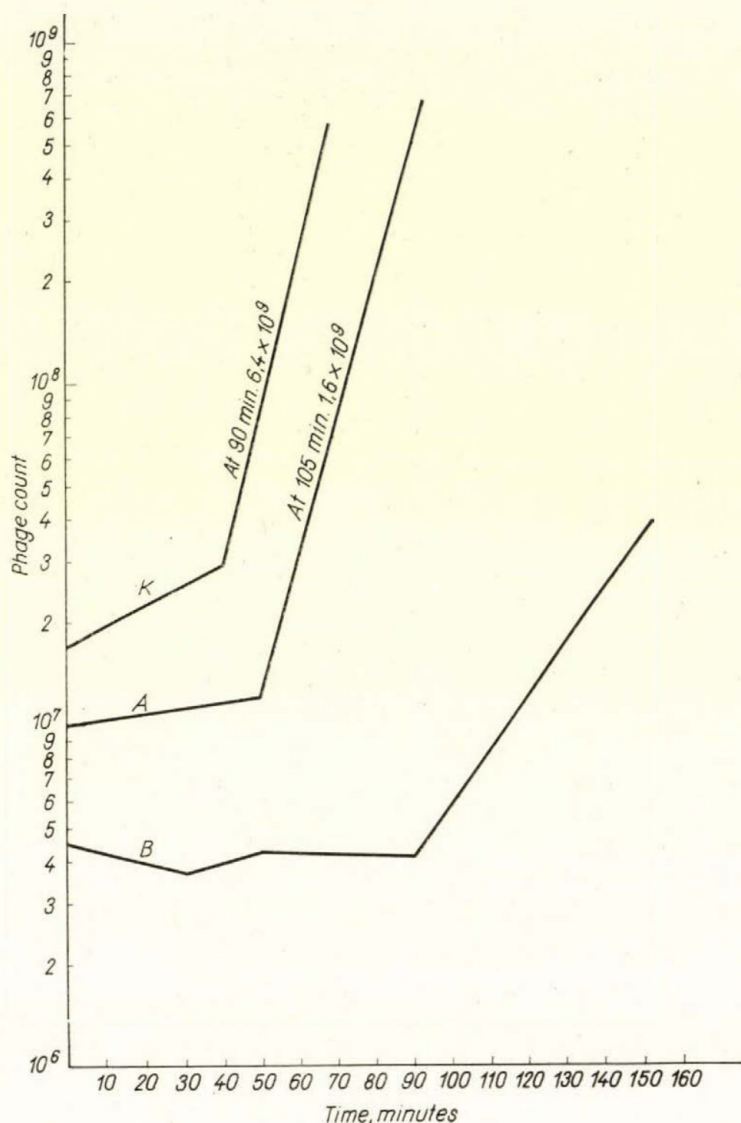


Fig. 3. Multiplication of *Cl. perfringens* phage following aeration of the growth mixture

A = aerated for 15 minutes

B = aerated for 50 minutes

K = control

intervals and incubated with aeration. The phage content of the mixtures both under aerobic and anaerobic incubation was assayed at intervals.

Fig. 2 illustrates the results of one experiment of this kind.

Curve K represents the phage titres in the growth mixture kept up to the end of the experiment under anaerobic conditions. Curves A, B, C and D

show the titres in the mixtures in which N_2 bubbling was changed for aeration at 15, 25, 35 and 45 minutes of incubation, respectively.

Fig. 2 also shows that the duration of preceding anaerobic incubation had determined whether phage multiplication stopped or continued in the aerobic milieu. If the anaerobic cultivation had not exceeded 25 minutes, a remarkable decline in the phage titre occurred (Curves *A* and *B*); if it was 35 minutes, the titre remained constant (Curve *C*). In the other hand, 45-minute anaerobic incubation was followed, in spite of the unfavourable aeration, by a rise in the phage titre nearly as high as shown by Curve *K* (Curve *D*). It should be noted that in the foregoing experiments the latency of the phage under investigation was found to be about 47 minutes.

The influence of preceding aerobic incubation on the latent period. Having demonstrated that aeration is a suitable method of stopping phage multiplication in the early stage of the growth cycle, it seemed desirable to investigate the mechanism of stopping. Adsorption mixture was prepared and diluted into growth mixture as described and the latter was distributed in three growth tubes. Two of the tubes were incubated with air, the third with N_2 gas. Aeration was stopped and replaced by introducing N_2 gas in tube *A* after 15, in tube *B* after 50 minutes of incubation. The phage content of each mixture was assayed at intervals. A typical experiment is demonstrated in *Fig. 3*. 0 minute means the beginning of anaerobic incubation, thus for curve *K* the beginning of incubation, while for Curves *A* and *B* the time of introduction of N_2 gas.

It is clearly seen that the initial titre was lower in experiments *A* and *B* than in the control (*K*) experiment. This, in agreement with the data shown in *Fig. 2*, suggests that preceding incubation of the growth mixture in an abundantly aerobic milieu led to some loss of phage activity. It is noteworthy in *Fig. 3* that Curves *A* and *B* show prolonged latent periods, and no rise in the phage titre during the latency. In the foregoing experiments the growth mixture was incubated aerobically.

It seemed of interest to study if a preceding aerobic incubation of the bacterium culture or of the phage preparation would lead to similar changes in the growth curve. These questions were investigated in the following experiments.

A *Cl. perfringens* culture like those used in the foregoing experiments was divided into two parts. One half was aerated at 37°C for 1 hour while the other was kept at room temperature in anaerobic milieu. The adsorption mixtures and the growth mixtures were prepared thereafter, and the growth mixtures were incubated with N_2 gas. The phage content of both mixtures was assayed at intervals. *Fig. 4* shows that aerobic incubation of the bacterial culture did not change the chief characteristics of the growth curve.

In another experiment the phage preparation was incubated aerobically at 37°C for 1 hour. The phage titre remained constant.

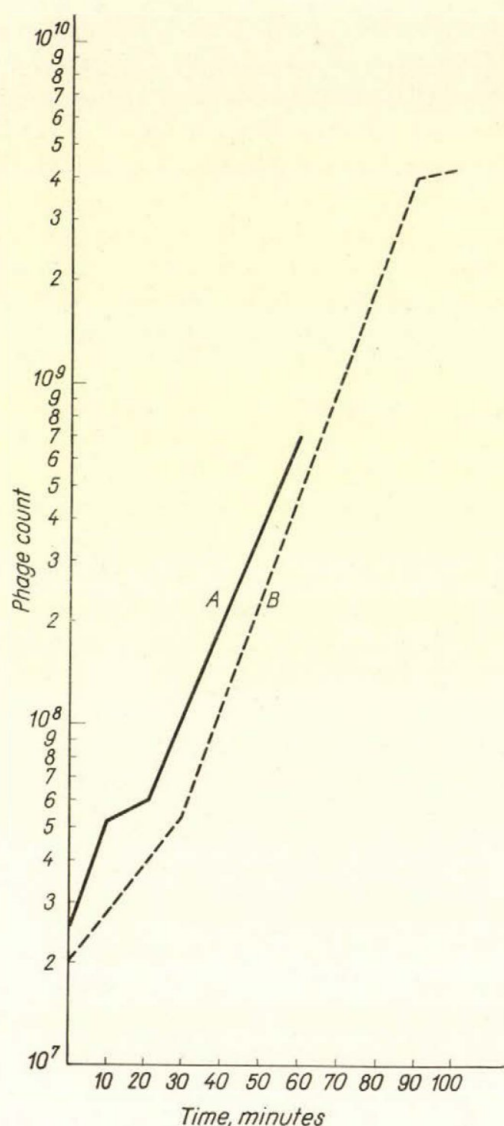


Fig. 4. Multiplication of *Cl. perfringens* phage after aeration of the bacterium culture.

A = growth in a culture previously aerated for 50 minutes
B = control

Discussion

It was demonstrated in our previous and the present investigations, in 6 experiments altogether, that the latent period of the 808/3a phage of *Cl. perfringens*, unlike that of the aerobic phages studied, is characterized by a

slow but considerable increase in the phage titre. The question has arisen whether this can be considered a true latency or a moderately steep initial sequence of the rise period beginning as early as at the 10th minute of incubation. In the present investigations we found that the rise period failed to come about if anaerobic incubation had been interrupted by introducing air at an early stage of the latency (at 35 minutes or earlier). Unlike this, if aeration had started at 45 minutes, *i. e.* at the end of latency, the rise period took place in spite of the unfavourable aerobic milieu. Based on these observations the first 45 minutes of the one-step curve, despite the slow increase in the phage count, can be regarded as a true latent period.

In studying the latent period of aerobic phages several authors have applied the method of "maturation inhibiton". DOERMANN [3] investigated the latency of the T3 phage by the so-called "premature lysis" method using KCN. He did not find matured phage particles during the first half of the latency; those appeared later, and when their count had attained a certain level, the usual "physiological" lysis occurred. In similar experiments carried out with the T5 phage KAY [2] observed hardly any phage production up to the 23rd minute of the latency lasting in whole 42 to 50 minutes. If KCN was added beyond this time, a reduced phage yield directly related to the time of adding cyanide was obtained. Our experiment illustrated in *Fig. 2* led to similar results, and aeration applied at the 35th minute or earlier proved to be an appropriate means to stop production of the anaerobic phage under study. It seems reasonable to distinguish two phases of the latent period. In the first phase, until about the 35th minute of incubation, there are no infective particles in the bacterial cells. In the second phase infective particles are present, and released thereafter even in an aerobic milieu.

The rise in the phage titre during the "latency" also needs explanation. Phage production in this period seems to be virtual. We suppose that our *Cl. perfringens* cultures consisted mainly of short chains of bacterial cells. Thus, the phage particles adsorbed to distinct members of the same chain gave rise to a single plaque. Streaming of nitrogen gas through the growth mixture possibly disrupted the chains step by step and so each infected cell became able to produce a separate plaque.

Growth mixtures having undergone a period of aeration showed a prolonged latency with constant phage count. A possible explanation of the altered latency is that the adsorbed particles while being aerated lose their growing capacity, and in the re-established anaerobic milieu the actually unadsorbed particles give rise to the prolonged curve. Another possible explanation is that the adsorbed particles penetrate the cells but aeration interrupts the growth cycle until the anaerobic milieu has been restored.

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Address of the author:

GYÖRGYI GÁSPÁR

State Institute of Hygiene, Gyáli út 2—6, Budapest IX, Hungary.

IMPRESSIONS OF *SH. SONNEI* COLONIES ON AGAR CULTURE MEDIA

By

B. SERÉNY

State Institute of Hygiene, Budapest

(Received February 2, 1960)

Summary. After removal of *Sh. sonnei* colonies grown on agar plates prepared with yeast extract, the culture medium shows formations reflecting the structure of colonies, which we have termed colony impressions. The impressions of colonies with phase II antigen alone appears as a round, whitish, turbid spot. Star-shaped colonies leave behind a clearly discernible star-shaped impression. No impression can be detected on the culture medium following removal of colonies consisting exclusively of organisms containing phase I antigen. The described phenomenon may to a certain extent be helpful in determining the antigen content of *Sh. sonnei* cultures. In the colonies containing phase II antigen the capacity for forming impressions is due to the microorganism's adhesion to one another and the agar surface.

In previous reports [15, 16] the appearance of star-shaped colonies in some *Sh. sonnei* cultures was discussed. While washing off star-shaped *Sh. sonnei* colonies from agar plates, our assistant V. DALOS found that he was unable to remove the star from the culture medium. This observation was the starting point of our investigations concerned with the impressions produced by *Sh. sonnei* cultures. The present report gives an account of the results obtained in various studies involving 400 *Sh. sonnei* cultures.

Materials and methods

Culture media. In addition to other conditions, the formation of colony impressions depends to a great extent on the quality of the culture medium. The yeast extract agar used in routine work at the *State Institute of Hygiene* has proved to be the most suitable; it is prepared by adding the following materials to 10 litres of boiling tap-water, amidst stirring:

Supernatant of yeast extract, 100 ml; Cell suspension of yeast extract, 5 ml; Agar-agar, 160—170 g; Sodium chloride, 30 g; Disodiumhydrophosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), 20 g; Peptone (Richter), 100 g.

The vessel with the mixture adjusted to 7.5 is then boiled for 45 minutes in an autoclave at 121° C (about 15 pounds pressure). The medium is left to cool in the autoclave until next morning, when the pure upper layer is decanted into half or one litre bottles and again sterilized at 121° C for 30 minutes.

Then 10 ml of sterilized 10 per cent glucose solution are added per litre of medium, amounts of 25 ml of which are then poured into Petri dishes 10 cm in diameter.

The yeast extract is prepared by mixing crude yeast with distilled water at a ratio of 1:1.2 heating this mixture to 60° C, and pouring it into bottles in which extraction takes place in an autoclave at 121° C for 30 minutes. The cells sedimented while the bottles sealed under aseptic conditions are left standing, and the supernatant are used at the above indicated ratio.

Agar plates prepared with beef extract are not suitable for the purpose of studying impressions; even when completed with 0.1 per cent glucose they rarely retain impressions. Nor do impressions appear with appreciable consistency on beef-peptone agar.

Inoculation. As emphasized in our previous reports, plates should be smeared with due care to obtain separate, isolated colonies.

Investigation of impressions. Adequately inoculated agar plates are incubated for two days at 37° C; then 10 to 15 ml of physiological saline are poured on them and they are left standing at room temperature for about 15 minutes. In this way most of the colonies are loosened and star-shaped formations can be clearly seen with the naked eye. For removal of the culture, the fluid in the Petri dish is drawn up into a 10 ml pipette and the whole surface is washed down. The fluid is then discarded and washing is repeated with 10 to 15 ml of physiological saline. After discarding this, 10 to 15 ml of physiological saline containing 5 per cent phenol are pipetted on the culture medium and left there for about 15 minutes. When the disinfectant solution is withdrawn, the plate is ready for investigation and may be stored for several weeks at room temperature.

The impressions of colonies examined against a black background are then clearly visible with the naked eye; minor variations may be discovered by inspection with a hand lens under transillumination.

The impressions can be stained. The turbid parts of the agar surface are taking the stains well while the unchanged areas stain either weakly or not at all. Informative contrast pictures can thus be obtained for instance with Löffler's methylene blue. When prior to phenol treatment approximately 10 ml of N/1 HCl solution containing 1 drop of Andrade's indicator are poured on the plate, the turbid agar surface assumes a bright red colour and even faint impressions are clearly shown.

Identification of *Shigella* cultures was carried out by the method described [15]; the results of cross exhaustion studies will be reported in another paper.

Results

Impressions produced by various colony types. *Sh. sonnei* leave mostly two kinds of impression. The round, evenly turbid impression (Fig. 1) of varying diameter (2 to 6 mm), corresponding to the size of the colony, is a whitish, turbid, and noticeably flat formation of roughly circular shape that hardly protrudes from the surface of the agar; its turbidity may vary in degree.

The star-shaped formation differs from the former by the presence around the centre of an irregular star-like pattern of varying size. Within the star the agar surface shows a slight or no change as compared to the peripheral area of the impression (Fig. 2).

Other forms are rare. Sometimes the round impression is hardly turbid or its margins are irregularly indented so that the impression appears as a blurred, turbid, irregular spot. In a few round, evenly turbid impressions the central part had cleared up to develop into a circular instead of a star-shaped form, producing a ring-like pattern (Fig. 3).

There is a certain, though not systematic, correlation between the type of colony and the structure of its impression. If prior to the removal of colonies their structure is determined in oblique light [15] and various parts of the colony are tested for antigen content by slide agglutination with exhausted sera, the following conclusions may be drawn.

Star-shaped impressions are formed by star-shaped colonies. The central radial part of such colonies is agglutinated by *Sh. sonnei* phase I serum, the peripheral material by phase II serum. The colonies showing marked refraction and no discernible pattern after incubation for one day must also be classed

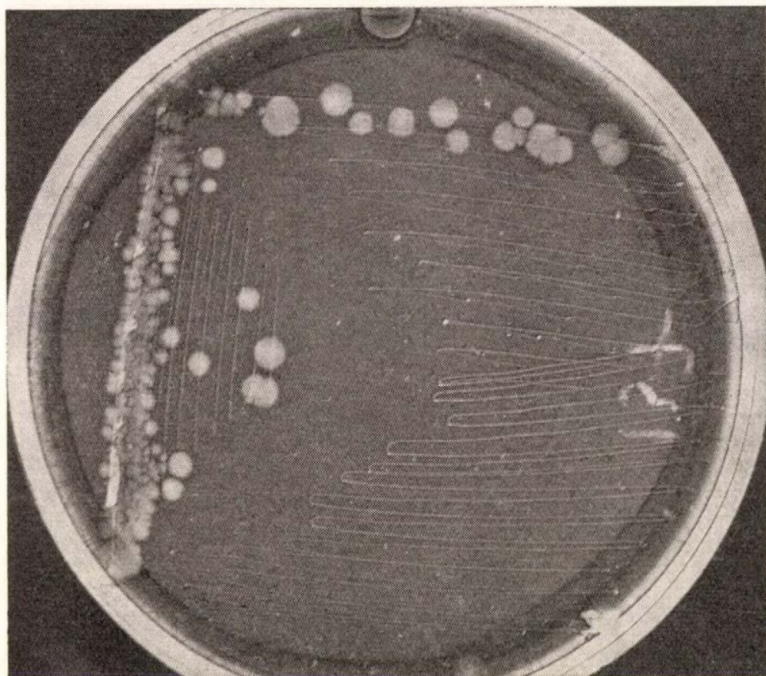


Fig. 1. Round evenly turbid impressions of colonies. Natural size. The photograph was made by placing sensitive paper directly under the agar plate and trans-illuminating with an enlarging bulb. Photograph by E. K. NOVÁK, State Institute of Hygiene, Budapest

with this group, if upon incubation for another day a faint yet definite radial structure may be detected in them when examined in the third plate position. Regarding antigen content, they agree with the star-shaped colonies. They leave behind star-like impressions; exceptionally, their impressions are ring-form.

The strongly refractive, homogenous colonies which display no structure after cultivation for two days and contain only phase I antigen according to cross adsorption tests, form no impressions whatever. After their removal the agar surface is found unchanged, glossy, transparent. Occasionally, slight amounts of phase II antigen can be demonstrated in this type of colony; in such cases slightly turbid spots of irregular shape are seen at their site.

Homogeneous, moderately refractive colonies containing phase II antigen alone have behind highly turbid impressions (Fig. 1).

Only five cultures of *Sh. sonnei* variant R were investigated. Their impressions were different from those of colonies containing phase II, appearing as whitish, highly turbid, irregular formations with indented contours.

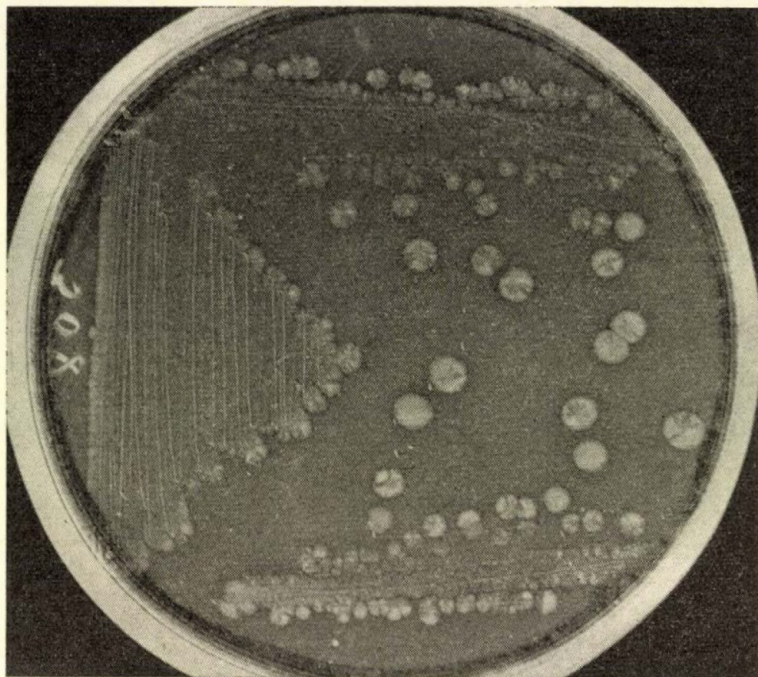


Fig. 2. Star-shaped impressions of colonies
Technique as in Fig. 1. Photograph by E. K. NOVÁK, State Institute of Hygiene, Budapest

Irregular development of impressions. The above general conclusions do not apply to every case. It happens that star-shaped or even moderately refractive homogeneous colonies are found to leave behind no impression. This was usually the case with dense, confluent cultures, but also with some adequately isolated colonies. In order to ascertain the incidence of this irregularity, 90 subcultures were grown simultaneously from star-shaped colonies. Upon incubation for one day, star-shaped, moderately refractive homogeneous colonies developed on every plate; however, after incubation for another day impressions were present on 83 agar plates only; from 7 plates the culture came off without leaving behind any trace. Approximately the same was the ratio of plates showing impressions and those appearing to be absolutely untouched, "sterile", when moderately refractive homogeneous colonies had grown simultaneously and two-day cultures were washed off. Hence the statements in the previous paragraph should be completed in that in about every tenth case the impression expected on the basis of colony morphology will fail to appear.

So far we have been unable to elucidate the origin of that phenomenon. Prolongation of the incubation time did not make the impressions to appear as a rule. Nevertheless, there was a certain connexion between incuba-

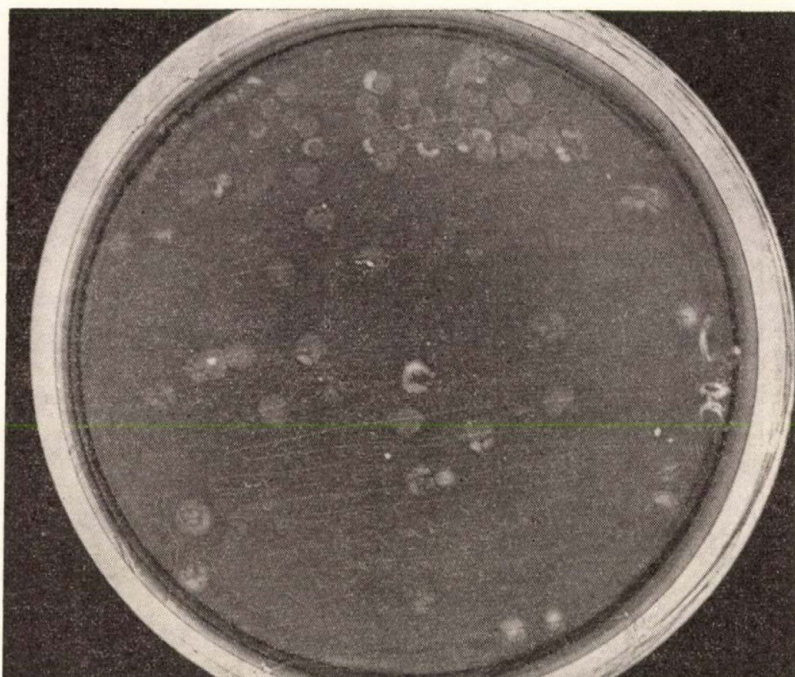


Fig. 3. Ring-like impressions

Technique as in Fig. 1. Photograph by E. K. NOVÁK, State Institute of Hygiene, Budapest

tion time and the formation of impressions. After one day's incubation, impressions were quite exceptional. On the other hand, in spite of incubation for 8 days no impressions were formed at the site of some star-shaped colonies. After prolonged incubation (e. g. for 10 days) though this had somewhat altered the picture, star-shaped impressions were still easily discovered (Fig. 4).

The way impressions are formed. In turbid impressions as well as in the turbid areas in star-shaped impressions, small protuberances, tiny papillar structures are seen next to each other under the microscope (Fig. 5); upon close inspection, longitudinal and transversal lines may be discerned on their surface. In the radial formation of star-shaped impressions these structures were absent, or quite small and certainly not common. When a star-shaped impression and then a slide are lightly touched with a piece of sterilized velvet fastened to a glass rod, the slide will be found to show a contact copy of the impression. Microscopic examination following staining of this preparation revealed that in the impression the place of the papillar structures had been occupied by masses of short, Gram-negative rods concentrated in islet-like foci, with no organism of a different shape among them. Thus the papillar structures consist of Gram-negative rods.

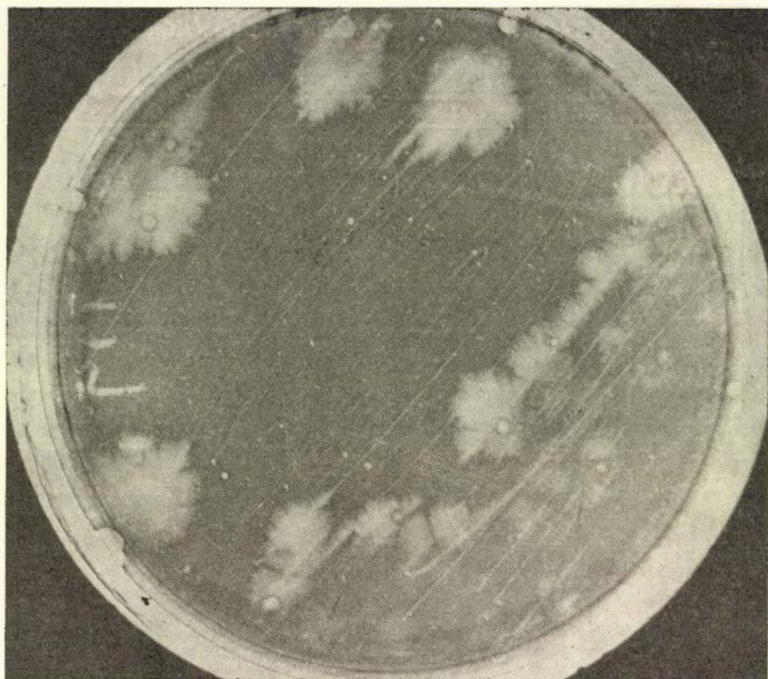


Fig. 4. Star-shaped impressions after incubation for 10 days
Technique as in Fig. 1. Photograph by E. K. NOVÁK, State Institute of Hygiene, Budapest

Similar results were obtained when cultures of *Sh. sonnei* had been washed several times in succession with sterilized physiological saline, and separate subcultures were made from both the impression and the unchanged areas of the plate. The former produced numerous *Shigella* colonies, the latter only a few.

The whole agar surface of colonies or colony-parts containing phase II antigen does not contribute to the formation of the impression, only the foci concentrated into islets do so. Before washing off, these foci may be distinguished under the microscope as irregular, highly refractive formations. They adhere to the surface of the agar more closely than do the other elements of the colony, therefore they remain on the culture medium despite removal of the colony by washing or by a loop, and produce an impression. In colonies and colony-parts possessing phase I antigen no such foci develop. Consequently no impression can be discovered at the site of colonies containing phase I alone, whereas star-shaped colonies leave behind star-shaped impressions.

The strong adherence to the agar of *Shigella* organisms concentrated into minute papillar structures is evidenced by the fact that the impression cannot be removed by washing repeated as many as 20 times, but new bacteria

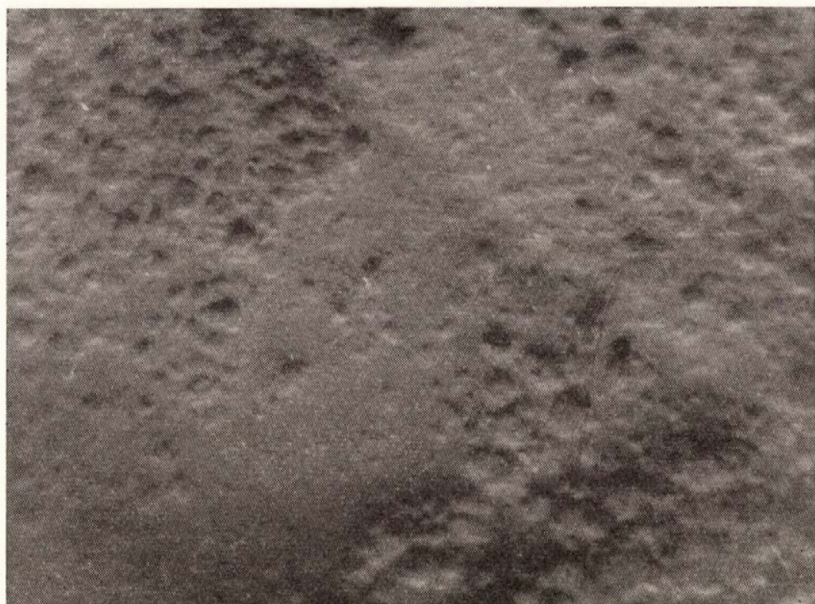


Fig. 5. The papillar structures become less towards the star's periphery. Magnification $\times 135$; condenser D 3. Photograph by E. K. NOVÁK, State Institute of Hygiene, Budapest

get into the washing fluid on every occasion. However, the papillar structures do not penetrate below the surface of the agar. By repeated washing and cautious separation with a loop, the impression can be removed in one piece; underneath, the agar surface is unchanged.

Discussion

The morphological, staining, antigenic and immunogenic differences shown by the dissociated variants of *Sh. sonnei* are well-known [1—4, 6—8, 10, 17]. Recently numerous data have been published to confirm the essential difference in virulence between phase I and phase II organisms [5, 9, 12, 13, 14, 18—21]. As regards the chemical structure of purified lipopolysaccharide protein antigen, no difference has been demonstrated between phase I and phase II [1].

In recent investigations the refraction of *Sh. sonnei* phase I has been found stronger than that of phase II organisms, and phase I star-shaped colonies or colony-parts are readily identified by examination in oblique light [15]. On desoxycholic acid (D) agar only phase I *Sh. sonnei* strains form brown stars [16].

In the present paper a hitherto unknown property of *Sh. sonnei* cultures has been discussed, viz. that the capacity of forming impressions of some of the organisms containing phase II is due to their adhesion to one another and to the agar surface.

The morphological variations and the relative number of different forms, together with the methods previously described [15, 16] make it possible to identify *Sh. sonnei* cultures and to estimate their antigen content.

Since the appearance of impressions is not consistently regular, the question presents itself whether impressions offer any information in addition to provided by the morphological picture of a colony appearing in oblique light. We believe that the chief advantage of the impressions lies in the simplicity of the procedure associated with their study; the whole culture and every one of its colonies lend themselves to prompt survey. Any hidden colony whose structure differs from that of the others is easily discovered. When examination of a one-day culture in oblique light is followed by incubation for another day and subsequent inspection of the impression, the latter result may serve as a control. The impressions offer the further advantage of being clearly demonstrable and suitable for storage over prolonged periods of time; on culture media dried for two or three days in an incubator they can even be forwarded by mail.

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Address of the author:

BÉLA SERÉNY

State Institute of Hygiene, Gyáli út 2—6, Budapest IX, Hungary.

THE INCIDENCE OF STAPHYLOCOCCI IN HOSPITAL PERSONNEL AND PATIENTS, AS STUDIED BY PHAGE-TYPING

By

HEDDA MILCH, MARIA EÖRSI* and M. BOGÁRDI

State Institute of Hygiene, Budapest, and Paul Heim Children's Hospital, Budapest

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Summary. As revealed by a survey of 1782 samples taken from patients and the nurses of a children's hospital, the incidence of *Staphylococcus* carriers was 10 per cent in the patients at the time of admission; during their stay in the hospital 71 per cent became *Staphylococcus* positive, 50 per cent within 6 days. Among the nurses 60 per cent were *Staphylococcus* carriers during the same period.

The incidence of the single *Staphylococcus* phage groups as listed in decreasing order was as follows: I, "mixed", III, and II. Of the strains 70 per cent could be typed. The incidence of not-typable strains was between those of groups "mixed" and III.

As to their distribution, group III was the most frequent both among the patients and the nurses, while group II strains were remarkably more frequent in the nurses and group III in the patients.

Using the method of phage-typing the ways of spreading were also followed. As compared to the others, group II strains were found to possess a moderate spreading activity. The role of patient to patient transfer was found to be of about the same importance as that from the nurses to patients or *vice versa*.

In 55 per cent of the nurses the phage-types were stable. Superinfections were more frequent among the patients than among the nurses.

As to the correlations between phage-type and antibiotic resistance, strains belonging to phage group III were found to possess the highest degree of resistance against all of the antibiotics examined.

As revealed by follow-up studies, phage-type and antibiotic resistance changed independently of each other. The sensitivity to antibiotics changed significantly more frequently in connection with the appearance of strains of new phage groups than with changes in phage sensitivity occurring within the same group.

The high incidence of hospital *Staphylococcus* infections has made it necessary to study the origin of infections, the ways of spreading and the virulence of strains isolated. Studies of this kind have been made possible by the method of phage-typing of *Staphylococci*. Both the results of several authors [1-9] such as ANDERSON and WILLIAMS, WILLIAMS *et al.* and others, as well as of ourselves revealed phage-typing to be the most effective tool in differentiating of *Staphylococci*.

The present report deals with the incidence of *Staphylococcus* carriers among the patients and nurses of a hospital and the phage-typing and antibiotic sensitivity testing of the strains isolated. The examinations were planned to give an answer to the following questions.

(i) The incidence of *Staphylococcus* carrier state in patients and nurses; relations between the incidence of carrier state and duration of hospitalization.

* Deceased.

- (ii) Phage-typing of the strains isolated and determination of the most frequent group together with an exact determination of the phage pattern.
- (iii) Distribution of phage groups in patients and nurses.
- (iv) Following up the ways of spreading of Staphylococci.
- (v) Examination of the stability and/or variability of the phage-types in the patients and the nurses.
- (vi) The possible correlations between phage group and antibiotic sensitivity.
- (vii) The correlation between the changes in phage-types and antibiotic sensitivity in repeated examinations.

Materials and methods

The studies were carried out at the *Infants' Department* of the *Paul Heim Children's Hospital, Budapest*. The period covered 3 months, from September to December, 1958. A total of 166 infants and young children were examined. The average number of nurses was 66 in this period; 51 worked continuously at the same department. From the patients and the staff 1266 resp. and 516 samples were taken. Only the coagulase positive *Staphylococcus aureus* strains were examined, these being exclusively accessible for phage-typing. To decide whether or not a strain could be phage-typed, coagulase production was considered the only indication as both pigment and haemolysin production turned out to be too much variable properties. Samples were generally taken from the nose, while in cases suspicious for Staphylococcal infection throat swabs, pus, faeces or other material were also examined.

Within a few days after the isolation the phage-typing was carried out by the method of WILLIAMS and RIPPON [2], using the phages listed below:

Group I: 29, 52, 52A, 79, 80;

Group II: 3A, 3B, 3C, 55, 71;

Group III: 6, 7, 42E, 47, 53, 54, 70, 73, 75, 77;

Group IV: 42D.

The phages were used either in a routine dilution (RTD) or in a 1000 times more concentrated suspension or undiluted. Undiluted phage was used if the strains were resistant at the RTD or the 1000 times more concentrated suspension. Part of the *Staphylococcus* strains was found to be sensitive only at the undiluted phage material. Identification by the latter method was always made by repeated examination of the same patient. For typing, horse meat bouillon agar containing Witte's peptone and 2 per cent of a 1 per cent calcium chloride solution was used. The agar plates were inoculated with a 2 hour bouillon culture of the bacterium strain examined, while the appropriate type phages were dropped on the surface of these cultures.

The antibiotic sensitivity tests were performed by the filtre-paper-disc method. The discs produced by the *Institute for Serobacteriological Production and Research "Human" (Budapest)* contained penicillin, streptomycin, chlortetracycline, oxytetracycline, chloramphenicol, neomycin, polymyxin or erythromycin.

To simplify the evaluation of the antibiotic sensitivity tests, the strains of moderate sensitivity were considered resistant. Thus in the pertinent Table the strains have been characterized either as sensitive or as resistant.

Results

The results of the survey are presented in *Table I*. A total of 1782 samples was examined. Positive results were obtained in 545 patients (43 per cent) and in 211 nurses (40 per cent). The total number of the strains isolated was 756.

Table I
Staphylococcus carrier survey

Designations	Patients	Nurses	Total
Number of examinations	1266	516	1782
Total	545	211	765
Carriers per cent	43.0	40.9	42.4

The results concerning the incidence of *Staphylococcus* carrier state are presented in *Table II*. Of the 166 patients examined, 118 (71.1 per cent) were positive, while among the 66 nurses 40 (60.6 per cent) were found to be positive.

Table II
Incidence of Staphylococcus carrier state in patients and nurses

Designation		Patients	Nurses	Total
		166	66	232
Number of persons examined	Total	118	40	158
	Per cent	71.1	60.6	68.1

The correlation between the period of hospitalization and the occurrence of positive carrier state are presented in *Fig. 1*. Of the patients 17 (10.2 per

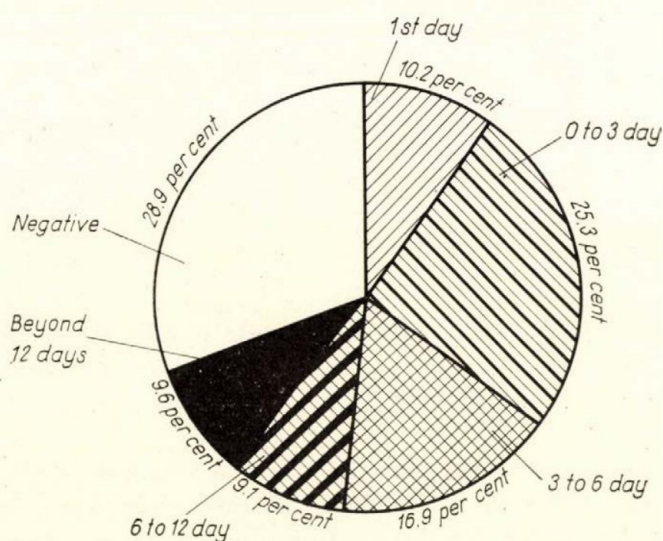


Fig. 1. Positive reactions of patients

cent) were positive on admission. This figure increased within 3 days to 42 persons (25.3 per cent). In the period from the 3rd to 6th day of hospitalization further 28 (16.9 per cent), within 6 to 12 days an additional 15 (9.1 per cent), and after 12 days further 16 patients (9.6 per cent) became positive. Only 48 patients (28.9 per cent) remained negative throughout. In 50 per cent of all the positive cases the coagulase producing *Staphylococcus aureus* strains could be isolated as early as within the first 3 days of hospitalization.

Table III

Distribution of typable strains in patients and nurses

Phage group	Patients	Nurses	Total	Patients	Nurses	Total
	Absolute number			Per cent		
Typable strains	393	139	532	72.1	65.9	70.4
Not-typable strains	152	72	224	27.9	34.1	29.6
Total	545	211	756	100.0	100.0	100.0

The results of phage-typing are summarized in *Table III*. The incidence of both typable and not-typable strains are presented for both the patients and the nurses. Out of a total of 756 strains examined 532 (representing 70.4 per cent of all the strains) were typable. Strains isolated from the nurses could be typed in 65.9 per cent, while those from the patients in 70.1 per cent.

Table IV

Distribution of typable strains per subjects

Designations	Patients		Nurses		Total	
	Total	Per cent	Total	Per cent	Total	Per cent
Typable strains	91	77	28	70	119	75
Not-typable strains	27	23	12	30	39	25
Total	118	100	40	100	158	100

In *Table IV* the incidence and the absolute number of typable strains is presented for the single persons examined. Among the 118 patients and 40 nurses typable strains were found in 91 (77 per cent) and 28 (70 per cent), respectively.

The distribution of typable *Staphylococcus* strains according to their phage groups is presented in *Table V*. The variations within the groups (phage-pattern) are not presented. The distribution according to phage groups was as follows: Of all the strains isolated 41 per cent belonged to phage group I.

Table V
Distribution of Staphylococci according to phage groups

Phage group	Number			Per cent		
	Patients	Nurses	Total	Patients	Nurses	Total
I.	154	64	218	39.2	46.0	41.0
II.	25	37	62	6.3	26.7	11.7
III.	60	3	63	15.4	2.1	11.8
IV.	—	—	—	—	—	—
Mixed	154	35	189	39.1	25.2	35.5
Total	393	139	532*	100.0	100.0	100.0

* $\chi^2_{[3]} = 58.0$; $P < 0.1\%$

Strains belonging to group "mixed", a type reported to occur seldom, were present in 35.5 per cent. In spite of its great spreading activity this group of strains did not cause disease except a slight angina. Group III strains had an incidence next to group "mixed".

The most frequently occurring phage-types as listed in decreasing order were in the patients, for group I: 80, 29, 52/52A/79/80; for group III: 53, 7, 47, 53/77; while in the nurses for group I: 29, 52/52A/80; and for group II: 3B/3C.

In Table V the data are presented separately for patients and nurses. The distribution of the phage groups was as follows. Strains belonging to group I were the most frequent in both the patients and the nurses. Strains of phage group III and mixed occurred pre-dominantly in the patients, while group II in the nurses. The difference in the incidence of groups II and III in patients and nurses were statistically significant.

Fig. 2 shows the incidence of the single phage-types. As to the spreading activity of strains belonging to the different phage groups, strains belonging to group II, in spite of having frequently been present in the nurses, did not spread to the patients. As an explanation, these strains might be supposed to possess a lower infectivity than those belonging to groups I and III. Strains belonging to group III were rare in the nurses but rather frequent in the patients. To decide whether the incidence found for the single phage groups represented the real situation or was only the result of making repeated examinations in the same persons, the results were evaluated as related to the persons examined as well. The results obtained by both methods were practically the same, as a proof of the weaker infectivity of group II strains.

Attempts were made to follow up the ways of spreading of the single groups. This is usually difficult except when an epidemic is caused by one predominant strain. We nevertheless succeeded in establishing that strains

belonging to group I were mostly carried over from the nurses to the patients, while those of group III from one patient to the other. Thus, with respect to the possibilities of epidemics, the role of patient to patient, and that of staff to patient transfer may be of equal importance.

It was remarkable that we regularly failed in demonstrating No. 80 type strain of group I (the so-called hospital epidemic strain), from the nasal secretions of the nurses, while it was very frequent in the patients. Some of these strains exhibited simultaneously a sensitivity to some other group I phage (*e.g.* 52/52A/80). ASHESHOW and RIPPON [10] and also ROUNTREE [11] reported in 1959 that they succeeded in converting the strain 80 by lysogenization into strain 52/52A/80 and *vice versa*. Also WAHL and FOUACE [12] observed transformation of types *in vitro*, occurring either spontaneously or produced by lysogenization. One might suppose similar changes to occur *in vivo*, as there is a very high incidence of different Staphylococci in nature. Thus the strains isolated from the patients might have originated from type 52/52A/80 type strains deriving from the nasal secretion of the nurses.

Table VI

Results of phage-typing of Staphylococci isolated from persons examined repeatedly

Phage-type	Number of persons examined			Average number of examinations per person		
	Patients	Nurses	Total	Patients	Nurses	Total
Unchanged	43	20	63	6.6	5.8	6.4
Changed within one group	9	5	14	5.5	8.6	6.6
Inter-group changes	33	5	38	6.1	8.4	6.4
Total	85	30	115	6.3	6.7	6.4

Some data concerning the stability of the phage-types were also obtained. The results of typings carried out on strains isolated from repeatedly examined 85 patients and 30 nurses are presented in Table VI. In 43 patients and 20 nurses no changes in the phage-type could be detected. Two sorts of changes were differentiated in the phage sensitivity of the Staphylococci isolated, *i. e.* (i) strains belonging to different types within the same phage group or (ii) those belonging to different phage groups, isolated on different occasions. No changes in the phage-type of the strains were observed in 50 and 75 per cent of the patients and the nurses, respectively. Changes within the same group were encountered in 9 strains from the patients and in 5 from the nurses. Variation from group to group was found in 33 strains from patients and in 5 from the nurses. There was no marked difference in the incidence of variation of sensitivity to phages within one group for strains repeatedly isolated from the patients and the nurses, while changing of the group itself was more frequent in the

patients than in the nurses. The reliability of the results was judged by relating the figures to the absolute number of repeated examinations. As the data presented in *Table VI* revealed no great variation in the average number of examinations, the results concerning the stability or variability of the phage types seemed to be reliable. The average number of examinations per person was 6.4. The low incidence of group to group variations in the nurses seemed to be proved further by the high number of examinations carried out in this particular group (8.4 per person).

Table VII

Percentual distribution of persons examined, according to changes in phage-type

Phage-type	Patients	Nurses
Unchanged	51	66
Changed within one group	10	17
Inter-group changes	39	17
Total	100	100

In *Table VII* the distribution of the phage-type changes in the persons examined are given in per cent. No changes in phage-type were found on repeated examination in 51 and 66 per cent of the strains from the patients and the nurses, respectively. Variations from group to group occurred in 39 per cent in the patients, while in 17 per cent in the nurses; apparently, this type of variation occurs more frequently in patients than in nurses (the values were on the border-line of significance). One might suppose that changes within one group resulted from changes in the phage sensitivity of the strains, while variations from group to group should be regarded as cases of superinfection. As revealed by our examinations, superinfections occurred more frequently among patients than among the healthy nurses. The detailed explanation of all the possibilities concerning the cause of this phenomenon can not be given here, thus we only mention that the sick organism appears to be more sensitive to superinfections. To elucidate the possible correlations between the primary diseases and the frequency of superinfections some additional studies are required.

Table VIII presents the correlation between sensitivity to antibiotics and the phage-types. Strains belonging to phage-type III were the most resistant against the antibiotics tested. There were remarkable differences in the sensitivity of the phage-types to penicillin, streptomycin, oxytetracycline, chlor-tetracycline and erythromycin. In phage group III, 100 per cent of the strains were resistant to penicillin, while in group II 51.6 per cent, and out of the strains belonging to the fairly frequent group I, only 85.7 per cent. Resistance to

Table VIII
Percentage of antibiotic resistant strains within the single phage groups

Antibiotic	Phage group					Total
	I.	II.	III.	Mixed	Nt*	
Penicillin	85.7	51.6	100.0	86.02	92.0	86.1
Chlortetracycline	72.9	66.1	90.4	77.4	80.6	77.2
Polymyxin B	54.1	48.3	58.7	59.6	62.9	58.1
Oxytetracycline	53.2	45.1	84.1	53.7	61.6	57.8
Streptomycin	38.9	35.4	74.7	44.6	72.2	53.0
Chloramphenicol	35.7	51.6	71.4	48.3	65.6	52.1
Erythromycin	10.5	12.9	41.2	17.2	37.8	22.8
Neomycin	16.0	25.8	23.8	18.2	17.6	18.7

* Not-typable

streptomycin was exhibited by 74.7 per cent of group III strains, 35.4 per cent of those belonging to group II, and 38.9 per cent of group I strains.

The highest antibiotic resistance was found for group III, the next for the untypable group, then for "mixed" and the least for phage groups I and II.

Table IX
*Variations of phage-type and antibiotic sensitivity of the strains,
as related to the subjects examined*

Phage-type	Resistance of strains to antibiotics		Total
	Unchanged	Changed	
A. Absolute number			
Unchanged	24	39	63
Changed within one group	5	9	14
Inter-group changes	6	32	38
Total	35	80	115
B. Per cent			
Unchanged	69	49	55
Changed within one group	14	11	12
Inter-group changes	17	40	33
Total	100	100	100

The correlations between antibiotic sensitivity and changes in the phage-type on repeated examination were also studied. The results are presented in *Table IX*, both in absolute numbers and percentages. Both, phage-type and

antibiotic sensitivity were found to be unchanged in 69 per cent and was changed in 49 per cent. When changes occurred within one phage group the sensitivity to antibiotics remained unchanged in 14 per cent and was changed in 11 per cent. Differences in antibiotic sensitivity were found to be significant when the phage group itself had changed. In 17 per cent of these cases the antibiotic sensitivity remained the same, while it changed in as much as 40 per cent.

Table X

*Distribution of patients examined,
as related to the variations in phage-type and resistance of the strains to antibiotics*

Phage-type	Resistance of strains to antibiotics		Total
	Unchanged	Changed	
A. Absolute number			
Unchanged	17	26	43
Changed within one group	5	4	9
Inter-group changes	6	27	33
Total	28	57	85
B. Per cent			
Unchanged	61	46	51
Changed within one group	18	7	10
Inter-group changes	21	47	39
Total	100	100	100

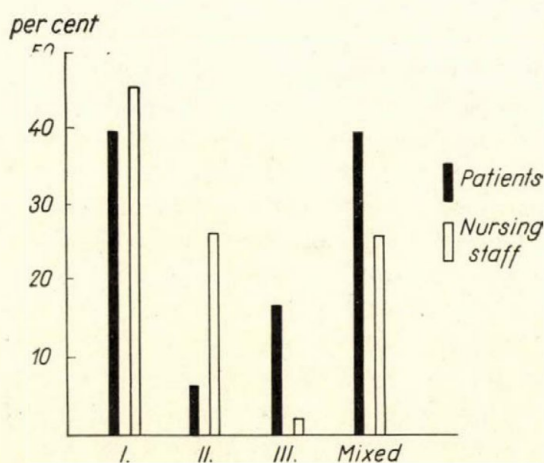


Fig. 2. Distribution of phage groups

Table X presents the data concerning the changes of phage-type and antibiotic sensitivity in the patients. It can be seen that antibiotic sensitivity was unchanged in 21, while changed in 47 per cent of the cases in which the phage group had changed. Thus for the patients the same degree of significance was obtained as for the summarized data in *Table IX*, i. e. the changes of the phage group were connected with a high incidence of changed antibiotic sensitivity. This observation supports the view that in such cases superinfection occurs rather than a variation of the strain.

Discussion

WILLIAMS [13] studied the incidence of *Staphylococcus* carrier state in adult persons by examining their nasal secretions. When carrying out phage-typing he found that certain hosts harboured strains of the same phage-type for several weeks.

ROUNTREE and BARBOUR [14] found a 52 to 71 per cent *Staphylococcus* carrier state when examining nasal secretions of hospital nurses. Most of the strains isolated belonged to the same phage-type.

KÁSS and VOGELSANG [15] observed coagulase positive *Staphylococci* in the nasal and throat secretions of infants and children in 73.6 per cent at admission and in 78 per cent at discharge.

RIPPON and VOGELSANG [16] detected 10 constant and 45 transitory *Staphylococcus* hosts among 67 hospitalized children; 12 of the patients were found to be free of contamination.

In our studies the incidence of *Staphylococci* in the nasal secretions of infants and children was remarkably lower than the incidence found by KÁSS and VOGELSANG. Nevertheless, after a certain period of hospitalization we found the same figures as the authors cited. As to the stability of the phage-types of the *Staphylococcus* strains present in hospitalized infants and children, it was found to be less stable than in the nasal secretion of the nurses.

Occurrence of different phage-type of *Staphylococci* in the same person on repeated examinations seems to have resulted mostly from superinfection. Such superinfections apparently occur more frequently in hospitalized children than in the healthy nurses. Further studies are required to decide whether these differences should be ascribed to differences in age or to those between a healthy and a sick organism.

When comparing our results to those of the other authors concerning the distribution of the phage-types (*Table XI*) found in different countries, it appears that while they found mostly strains belonging to phage group III in both the patients and the nurses, we could mostly isolate strains of phage group I.

Table XI

Phage group distribution of Staphylococcus strains according to different authors (Groups I to III)*

Authors	Number of strains	Per cent of type-able strains	Phage group			Origin of the strain
			I	II	III	
VOGELSANG (Denmark)	858	62.9	4.0	9.5	49.4	Nursing staff
GOULD and McKILLOP (England)	460	62.0	25.0	15.0	23.0	Healthy adults (Medical students)
Present studies (Hungary)	139	65.9	46.0**	26.7	2.1	Nursing staff
McLEAN (Australia)	262	53.0	5.0	6.5	55.7	Hospitalized patients
ROUNTREE and RHEUBEN (Australia)	96	94.8	47.9**	26.0	14.6	"
FUSILLO <i>et al.</i> (USA)	485	73.0	7.6	2.5	62.9	Clinical samples
Present studies (Hungary)	393	72.1	39.2**	6.3	15.4	Hospitalized patients

* According to H. Brandis [17]

** Type 80 predominant

The results of ROUNTREE of Australia revealed a high incidence of phage-type I strains. Moreover, just like in our material, type 80 of the same group was the predominant one. Some of our unpublished data [18] obtained in examinations on a wider scale also revealed a remarkable predominance of phage group I. Similarly as with the *S. typhi* and *S. paratyphi B* phage-types, there must be a special geographical distribution of *Staphylococcus* phage-types.

VOGELSANG [19] when examining the correlation between phage-type and antibiotic resistance, found that strains of group III were in 68 per cent resistant to penicillin, while those of group I in 35 per cent and those of group II in 15 per cent. Similarly, the highest incidence of penicillin resistance was found in strains of group III by ROUNTREE [4], FUSILLO *et al.* [20], WALLMARK [21], and BARBER *et al.* [22]. Nevertheless, BARBER *et al.* [23], RUYS *et al.* [24], and SCHMIDT and LENK [25] observed occasionally an overwhelming penicillin resistance in phage groups I and II, and among strains untypable by phages. VÁCZI *et al.* [26] found the highest incidence of antibiotic resistance among the untypable strains.

In our own examinations, resistance to penicillin amounted to 100 per cent in group III, 92 per cent in the untypable group, 86 per cent in the "mixed" group, 85.7 per cent in group I, and 51.6 per cent in group II. The highest resistance against all the antibiotics tested was found in phage group III.

If it is supposed that the wide spreading of a particular *Staphylococcus* strain is an important factor in the development of antibiotic resistance, one

would expect the highest resistance in strains belonging to phage group I. As, on the contrary, the highest resistance was found in group III, one is inclined to suppose that there is no direct correlation between the antibiotic resistance and the high incidence of a particular strain.

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Address of the authors:

HEDDA MILCH, State Institute of Hygiene, Gyáli út 2-6, Budapest IX, Hungary.

MHÁLY BOGÁRDI, Paul Heim Children's Hospital, Üllői út 82, Budapest VIII, Hungary.

STUDIES ON THE AMINO ACID COMPOSITION OF THE CELL WALL OF *E. COLI* O:111 STRAINS WITH DIFFERENT ANTIBIOTIC SENSITIVITY

By

L. VÁCZI, M. FODOR, A. RÉTHY and I. HOLLÓS

*Institute of Microbiology, University Medical School, Debrecen, and State Institute of Hygiene,
Budapest*

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Summary. The amino acid composition of the cell wall of *E. coli* strains has been studied. The examinations were performed with an antibiotic sensitive strain and with strains that had developed antibiotic resistance *in vivo* and *in vitro*. Cell wall preparations were obtained by the use of different methods. The phases of preparation were checked electron-microscopically.

In the cell wall of *E. coli* O:111 strains 17 various amino acids were demonstrated. Of these glutamic acid, alanine, aspartic acid, the leucines and also diaminopimelic acid were found to occur in the largest proportion.

There was no significant difference between sensitive and resistant organisms as to the amino acid constitution of the basal structural elements of the cell wall. The difference between the results of various preparation methods as to the diaminopimelic acid content of the cell wall of sensitive and resistant organisms may be due to changes in the surface structure of the cells.

The composition and structure of the bacterial surface plays an important part in the biological reaction to environmental effects. In previous investigations it has been shown that the surface of *E. coli* O:111 strains which acquired resistance *in vitro* or *in vivo* to antibiotics, is considerably richer in lipids than that of the sensitive strains [1, 2]. A similar difference has been shown between the composition of the surface of antibiotic-sensitive and polyresistant *Staph. aureus* strains [3]. Considering these findings, it seemed necessary to determine whether there was a difference between the amino acid content of the cell wall of *in vivo* and *in vitro* resistant *E. coli* O:111 strains, and whether this difference might be associated with morphological changes characterizing the strains resistant *in vitro* and with their altered biological properties. In the examinations a special attention was paid to the alpha-epsilon-diaminopimelic acid (DAPA) content of the cell wall, as according to WORK [4] and WORK and DEWEY [5] and others this substance plays an important part in the basal structure of the cell wall.

Materials and methods

Test organisms. (a) *E. coli* O:111 strain 7 E. Sensitive to streptomycin, chloramphenicol and polymyxin; moderately sensitive to terramycin and aureomycin; resistant to penicillin and erythromycin;

(b) *E. coli* O:111 strain 111. Resistant to penicillin, streptomycin, chloramphenicol, terramycin, aureomycin and erythromycin; sensitive to polymyxin;

(c) *E. coli* O : 111 strain 9 R. Trained *in vitro* to resistance by serial subcultures in broth containing gradually increasing concentrations of chloramphenicol. Resistant to penicillin, chloramphenicol, terramycin, aureomycin and erythromycin; sensitive to streptomycin and polymyxin.

Preparation of the cell wall. Method 1. Tryptic digestion after exposition to heat. The procedure was a combination of the methods described by WEIDEL [6] and SALTON and HORN [7, 8]. The strains were grown in shake cultures at 30° C for 48 hours. The cultures were centrifuged and washed with distilled water. The bacteria were then resuspended in an equal part of distilled water. This suspension was sprayed into continuously stirred boiling distilled water and subsequently placed in ice water.

The suspension was then mixed with an equal part of pH 8.2 ($\text{NH}_4\text{OH} + \text{NH}_4\text{Cl}$) Michaelis buffer. To each 100 ml of the mixture 1 g trypsin and 0.2 g streptomycin were added. Digestion lasted for 4 days at 37° C. The pH of the mixture was corrected daily. Then a differential centrifugation was performed (3 times for 5 minutes at 3000 r. p. m. and once for 5 minutes at 4500 r. p. m.). Finally the cell walls were sedimented for 60 minutes at 7000 r. p. m. and washed twice with distilled water. The degree of purity of the cell wall preparations was approximately 90 per cent.

Method 2. Alkaline preparation according to KANDLER *et al.* [9]. One part of washed bacteria was incubated in approximately ten parts of *N* NaOH at 60° C for 3 hours then at room temperature for 16 hours. After the addition of an equal part of distilled water the mixture was centrifuged for 1 hour at 7000 r. p. m. and subsequently washed 3 times with distilled water.

The various phases of the procedures and the purity of the cell wall preparations were continuously checked by means of light and electronmicroscopic examinations. Unfixed smears were covered with a coverslip bearing a drop of diluted carbolfuchsin and examined under the microscope with oil-immersion. Intact cells gave a definitely contoured cell wall staining, disrupted cells were characterized by a broken up appearance of the cell wall. In contrast to the faint pink colour of the cell wall remnants, the protoplasm stained red.

For the electronmicroscopic examination the bacteria were transferred onto Formvar films. The preparations were shadowed with gold or were studied unshadowed.

Amino acid analysis. The cell wall suspension was washed with distilled water, treated with 82 per cent ethanol and dried at 102° C. To one part of the dry material 150 parts of 6 *N* HCl (w/v) were added, the mixture was hydrolysed for 24 hours in a sealed bomb tube at 105° C and then dried by evaporation in a water bath.

The dry material was mixed with distilled water and the evaporation was repeated 3 times. Finally the residue was redissolved in 1 ml of distilled water and its alpha-amino nitrogen content was determined by polarographic analysis. As a supporting electrolyte copper phosphate was used as described by MARTIN and MITTELMANN [10] at pH 9.5 as recommended by LINDNER [11]. For chromatography the stock solutions were diluted so as to correspond to 11.66 μg alpha-amino nitrogen or to multiples of this quantity.

For the detection and identification of amino acids two-dimensional chromatograms were run, using as solvents phenol : water : (NH_3) collidine resp. *n*-amylalcohol : pyridine : water = 80 : 20 : 15 (w/w). For the quantitative analysis descending chromatography with various solvents was applied as follows.

(1) For the determination of lysine, histidine, arginine, tyrosine and phenylalanine, *n*-butanol: glacial acetic acid: water — 4 : 1 : 1 and 4 : 1 : 5 (v/v) on Whatman No. 1 paper at 22° C.

(2) For the determination of aspartic acid, DAPA, glutamic acid, serine, glycine, threonine and alanine, phenol saturated with pH 12.0 McFarren buffer on Schleicher-Schüll 2043/b paper at 22° C.

(3) For the determination of proline, valine, methionine and iso-leucine and also of the leucine-isomers together, benzylalcohol: *n*-butanol — 1 : 1 (v/v) saturated with pH 8.4 McFarren buffer, on Schleicher-Schüll 2043/b paper.

(4) For the determination of DAPA, methanol: water: 10 *N* HCl: pyridine — 80 : 17.5 : 3.0 : 20 (v/v) on Whatman No. 1 paper, at 22° C. As a standard, a freshly-prepared stock solution of LL + meso-DAPA was used.

As recommended by LINDNER [12], in the quantitative analyses purified casein hydrolysate was employed as a standard. The spots obtained with the phenol-containing solvent were marked under UV light, then submitted to polarographic analysis. Other amino acids were converted into "Wieland's dye-complex" and estimated photometrically [12]. Cystine was determined by BRDICKA's polarographic method [13]. In the investigations a V 301 Heyrovsky polarograph and a Joben Yvon spectrophotometer were used.

Results

Cell wall preparations were made from strain 7E (sensitive to antibiotics), strain 111 (polyresistant), and strain 9R (chloramphenicol resistance induced *in vitro*). The various phases of the procedures and the purity of the cell wall preparations were checked in smears stained with diluted carbolfuchsin and in electronmicroscopic preparations shadowed with gold. It was striking that on heating the *in vitro* resistant *E. coli* O : 111 strain was more resistant to lysis than the sensitive or *in vivo* resistant strains. Under the light microscope

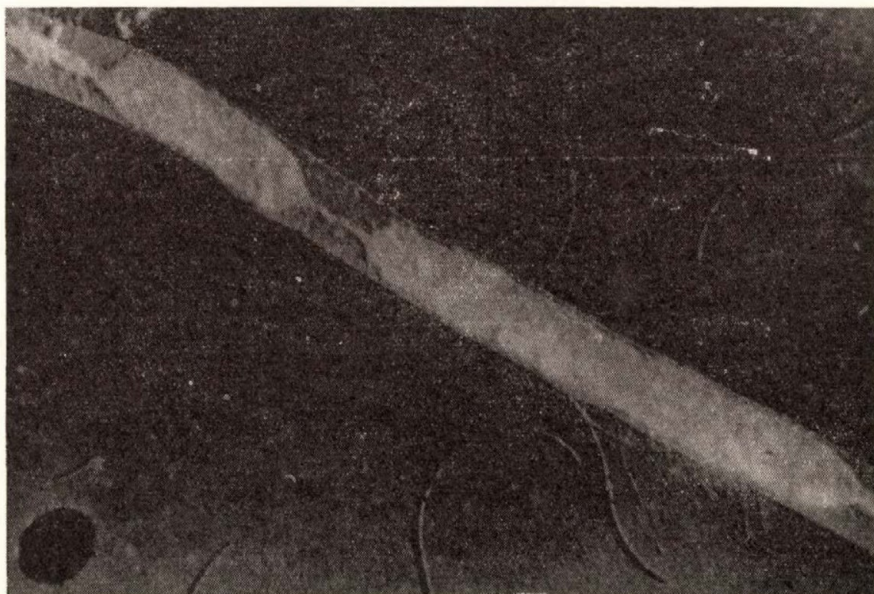


Fig. 1. Electronmicrograph of the *in vitro* resistant *E. coli* O : 111 strain 9R. Magnification approx. $\times 29\,000$. Shadowed with gold

and in electronmicrographs no morphological differences were demonstrated between the sensitive organisms and those resistant *in vivo*; the strain trained *in vitro* to resistance produced large numbers of filaments. Each of the latter forms corresponded to more than one cell, as without the complete separation of the body, protoplasm was clearly divided.

Fig. 1 presents the electronmicrograph of the *in vitro* resistant strain 9R in a preparation shadowed with gold at a magnification of approximately 29 000. The outer cell wall of the bacterium is intact and encloses the protoplasm without interruption. The protoplasm itself is connected by thin bridges.

The cell wall preparations from the various strains showed no morphological differences. The trypsin-lysed preparations were, however, not so pure as

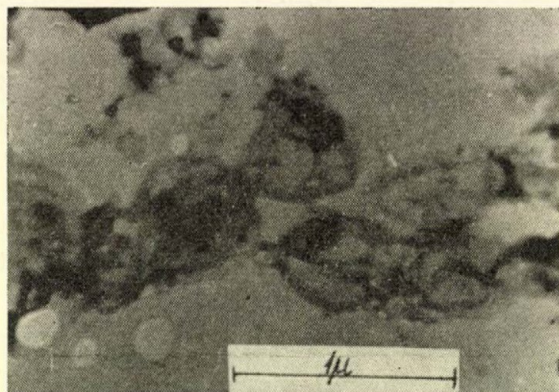


Fig. 2. Cell wall preparation obtained by tryptic digestion after heating. Unshadowed.
Magnification approx. $\times 29\,000$

those obtained by treatment with alkali. The latter procedure, when lasting more than 6 hours caused an increased disintegration of the basal structure of the cell wall. In such preparations only fragments of the cell wall could be demonstrated. Thus treatment with alkali should continuously be controlled by means of a light microscope and in electronmicrographs.

The electronmicrographs illustrating the cell wall preparations are presented in *Figs. 2, 3 and 4.*

The amino acid composition of the proteins in different cell wall preparations was determined by chromatography. *Table I* shows the percentage distribution of the various amino acids found in preparations obtained by tryptic digestion after heating. The alpha-amino nitrogen content of each preparation was equivalent to that of 100 μg casein.

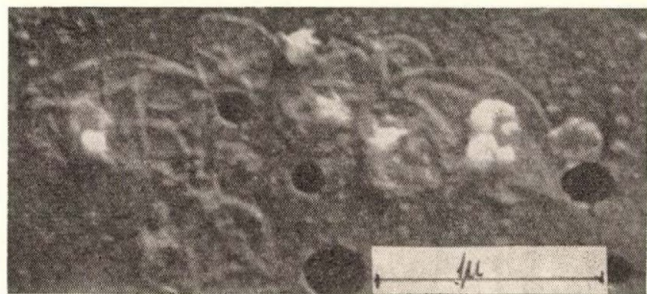


Fig. 3. Cell wall preparation obtained by tryptic digestion after heating. Shadowed with gold.
Magnification approx. $\times 29\,000$



Fig. 4. Cell wall preparation obtained by treatment with alkali. Slight protoplasmic contamination within the spread-out cell wall. Shadowed with gold. Magnification approx. $\times 29\,000$

According to *Table I*, glutamic acid, alanine, asparagine and leucines were the main amino acid components of the cell wall. They amounted to 43 to 46 per cent of the determinable amino acids. Glycine, lysine, tyrosine, serine, threonine, arginine, phenylalanine and valine occurred in smaller quantities, altogether in 44 to 46 per cent of the total, with glycine in the greatest amount and proline, methionine, histidine and cystine in less than 3 per cent. The significance of these amino acids was especially difficult to estimate, as they might have originated from impurities and not from the cell wall. This seemed to be confirmed by the observation that increased tryptic digestion resulted in a parallel decrease in the quantity of these amino acids. As to the distribution of amino acids in the cell wall proteins of bacteria with various antibiotic sensitivity a difference was only shown in the occurrence of DAPA. The significance of this will be discussed later.

Table II presents the amino acid composition of the cell wall proteins after lysis by alkali. It is clear that, in comparison with the former method, this procedure yielded more reliable information about the basal structure of the cell wall and of the amino acid composition of the cell wall proteins of *E. coli*.

According to *Table II*, apart from the DAPA content, there was no difference between the *E. coli* O:111 strains with various antibiotic sensitivity. The preparations obtained by both methods were characterized by an amino acid pattern practically corresponding to that of the complete proteins. This

Table I

*The amino acid composition of the cell wall of E. coli
0 : 111 strains with different antibiotic sensitivity*

Tryptic digestion

Amino acid content given as percentage of 11.6 μg alpha-amino nitrogen*

Amino acids	Strain 7 E sensitive	Strain 111 in vivo resistant	Strain 9 R in vitro resistant
1. Glutamic acid	13.7	14.5	14.4
2. Alanine	10.1	10.3	9.4
3. Leucines	10.0	12.0	10.1
4. Aspartic acid	10.0	9.6	9.3
5. Glycine	7.4	7.0	6.8
6. Lysine	6.5	6.1	5.9
7. Tyrosine	6.4	7.0	6.0
8. Serine	6.4	6.1	6.7
9. Threonine	6.4	6.1	5.8
10. Arginine	5.5	5.3	5.0
11. Phenylalanine	5.4	5.2	5.1
12. Valine	3.6	3.5	3.4
13. Proline	2.7	2.6	2.5
14. Methionine	1.8	1.8	1.7
15. Histidine	1.8	1.7	1.7
16. Cystine	0.5	0.4	0.4
17. Diaminopimelic acid	1.8	0.8	5.8
	100.0	100.0	100.0

* Means of 3 parallel determinations. Limits of error $\pm 15\%$.

is in sharp contrast with the composition of the cell wall of Gram positive bacteria, which contain a simple polypeptide made up of only a few amino acids. As regards the DAPA content of the examined strains, a considerable difference was found between the results of the two methods. The alkaline hydrolysates contained much more DAPA than those obtained by heating and tryptic digestion. The most definite difference was noted in bacteria resistant *in vivo*.

Considering the significance of DAPA, it seemed necessary to elucidate whether there really existed a difference between antibiotic resistant and sensitive strains in this respect or the finding was merely due to the different methods of preparation. In subsequent experiments the two cell wall preparation methods were combined. After treatment with alkali for 3 or 6 hours the cell walls were digested with trypsin. In the determination of the amino acid composition of these preparations special attention was paid to changes in the DAPA content.

Table II

*The amino acid composition of the cell wall of E. coli
O : 111 strains with different antibiotic sensitivity*

Alkaline cell wall preparation
Amino acid content given as percentage of 11.6 μ g alpha-amino nitrogen*

Amino acids	Strain 7E sensitive	Strain 111 in vivo resistant	Strain 9R in vitro resistant
1. Glutamic acid	13.6	14.3	14.4
2. Alanine	10.2	9.6	9.6
3. Leucines	10.2	10.3	10.3
4. Aspartic acid	8.5	7.2	7.2
5. Glycine	6.0	6.3	6.3
6. Lysine	6.8	6.3	6.3
7. Tyrosine	6.7	7.2	6.3
8. Serine	6.0	6.4	5.6
9. Threonine	6.0	5.6	5.6
10. Arginine	5.5	4.8	4.8
11. Phenylalanine	5.6	4.8	5.6
12. Valine	3.3	3.2	3.2
13. Proline	2.5	2.4	2.4
14. Methionine	1.6	1.6	1.6
15. Histidine	1.6	1.6	1.6
16. Cystine	0.4	0.4	0.4
17. Diaminopimelic acid	5.5	8.0	8.8

* Means of 3 parallel determinations. Limits of error $\pm 15\%$.

Table III

*Diaminopimelic acid content of the cell wall of sensitive and resistant E. coli O : 111
strains according to various methods of preparation*

DAPA content given as percentage of 11.6 μ g alpha-amino nitrogen*

Method of preparation	DAPA content	
	Sensitive strain	Resistant strain
Treatment with alkali for 3 hrs.	6.0	10.0
Treatment with alkali for 6 hrs.	8.0	13.6
Treatment with alkali for 3 hrs. then tryptic digestion	13.0	17.5
Treatment with alkali for 6 hrs. then tryptic digestion	19.0	17.0
Treatment with heat, alkali and trypsin	10.5	1.5

* Means of 4 parallel determinations. Limits of error $\pm 10\%$.

From Table III it is clear that the DAPA content of the cell wall increased with the prolongation of alkaline treatment. After tryptic digestion the remaining cell wall fraction contained even more DAPA. The observation that after the decomposition of the more superficial protein components DAPA made up $\frac{1}{5}$ to $\frac{1}{6}$ of the total quantity of amino acids was considered a proof of DAPA being a real constituent of the basal structure of the cell wall. Accordingly, no difference in DAPA content could be demonstrated between sensitive and resistant strains. Treatment with alkali and trypsin resulted only in a slight change in the DAPA content of the cell wall of resistant organisms, while the same procedure caused a manyfold increase of this amino acid in sensitive bacteria. This finding may be explained by a different chemical binding of DAPA in the two types of organism. The results obtained with heat-treated preparations also showed the difference in chemical binding, as in these experiments the *in vivo* resistant strain contained considerably less DAPA than the sensitive organism did. On heating, the resistant strains left a considerable part of their DAPA content in the supernatant, whereas sensitive bacteria did not. The *in vitro* resistant strain 9 R, which exhibited a morphological change and was more resistant to lysis than others, contained three times as much DAPA in the cell wall as the sensitive strain.

Discussion

The development of up-to-date preparation methods has resulted in a considerable progress of the chemical investigation of the bacterial cell wall. Valuable data have been obtained as to the composition of this part of the cell, which, regardless of its limited metabolic activity, plays an important role in the defence of the cell. The cell wall of bacteria generally consists of materials which, in living tissues occur as elements concerned in supporting other structures. The special function of the cell wall is evidenced by the findings that the extraordinary constituents of the bacterial cell, such as DAPA, muramic acid, and the D-isomers of amino acids occur almost exclusively in this part of the organism. In contrast to the cell wall composition of Gram-positive bacteria, that of Gram-negative organisms has been less extensively investigated. The explanation for this and the contradicting data in the literature is that preparations of suitable purity are difficult to obtain from the latter. Opinions generally agree that unlike the cell wall of Gram-positive bacteria that of Gram-negative organisms contains all the amino acids present in proteins. According to WEIDEL and PRIMOSIGH [14], minor particles separated from the cell wall of Gram-negative bacteria by phage action are similar in composition to the cell wall of Gram-positive organisms.

Taking into consideration the importance of DAPA, it has been tried to elucidate whether there is a difference between the amino acid pattern of

the cell wall of antibiotic-sensitive and -resistant *E. coli* O:111 strains and whether this difference ought to be attributed to a structural change. In order to obtain reliable data, the organisms were studied in parallel examinations by various preparation methods. In contrast to previous investigations, instead of measuring the dry weight, the determination of amino acid components was carried out in hydrolysates containing equivalent quantities of alpha-amino nitrogen. Of the amino acids present in the cell wall of the examined strains glutamic acid, alanine, the leucines, aspartic acid and DAPA occurred in the highest proportion. Methionine, histidine and cystine made up 1.6 per cent or even less.

It was striking that the main constituents of the cell wall of Gram-positive organisms, glutamic acid and alanine should occur in the highest proportion in the cell wall of Gram-negative bacteria as well. The fact that increased treatment with alkali caused an increase of the DAPA content simultaneously with a proportional decrease of the other amino acids, makes it probable that the basal structure of the cell wall of *E. coli*, similarly to that of Gram-positive bacteria, contains few amino acids and the distribution of these is very similar in the two types of organism.

In the present investigations no significant difference was demonstrated in the amino acid content of the cell wall of antibiotic-sensitive and of *in vivo* and *in vitro* resistant *E. coli* strains. This finding agrees with those of CUMMINS and HARRIS [15] who investigated the amino acid composition of the cell wall of antibiotic-sensitive and -resistant *Staph. aureus* strains.

Cell wall preparations of sensitive and resistant *E. coli* strains obtained by the same method showed a difference in amino acid composition. This pointed to a structural difference in the cell wall proteins of the two biologically different organisms. The same was confirmed by the significant difference in the DAPA content of sensitive and resistant strains which had been submitted to treatment with heat, alkali and trypsin. The slight differences in DAPA content of various strains yielded by the two methods of preparation were also suggestive of a structural difference.

SALTON and SHAFI [16] showed that penicillin caused changes in the surface structure of bacteria. Similar changes were observed by ISHIMOTO, SAITO and ITO [17]. Our own investigations revealed a difference in the cell wall proteins of biologically different *E. coli* strains. Accordingly, antibiotic-sensitive and -resistant strains differ not only in the composition but also in the structure of the surface constituents. Further investigations regarding this problem are in progress.

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Address of the authors:

LAJOS VÁCZI, MIHÁLY FODOR, ANTAL RÉTHY

Institute of Microbiology, University Medical School, Debrecen, Hungary

IVÁN HOLLÓS

State Institute of Hygiene, Gyáli út 2—6, Budapest IX, Hungary.

PREPARATION OF PRIMARY HUMAN AMNION TISSUE CULTURES

By

ILONA BÉLÁDI, ESZTER KUKÁN, I. MÉCS and E. SZÖLLÖSY

Institute of Microbiology, University Medical School, Szeged

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Summary. The preparation of amniotic tissue cultures is discussed on the basis of experience gained with 252 amnion membrane cultures. On the comparison of trypsinization at 37° C and 33° C, respectively, the former turned out to be more advantageous. Attempts were made to decide whether morphological examination and trypan blue staining were suitable methods to yield information about the viability of the cells for preparing tissue culture. The influence of different sera on the growth of cultures was also examined. The preparation of some preliminary tube cultures was found to be the most convenient method for deciding the cultivability of the cells trypsinized. Until the evaluation of the preliminary cultures the original cell suspension was maintained in culture medium containing glutamine. The storage did not alter the viability of the cells. By this method much superfluous work could be avoided.

The first successful attempt to prepare human amnion tissue culture by trypsinization was made by ZITCER [1]. His method has been widely used and was modified by other investigators [2, 3, 4]. The use of this tissue culture is especially advantageous in laboratories where no monkey tissue culture is available, the susceptibility to human pathogenic viruses of the amniotic culture being similar to that of monkey kidney tissue and its production being less expensive. Consequently, it was employed also in our laboratory. In this paper we wish to give an account of our experience with 252 amniotic membranes.

Materials and methods

1. Hanks' solution without NaHCO_3 ;
 2. Sodium bicarbonate—carbonic acid buffer; 4.5 per cent NaHCO_3 bubbled with CO_2 , pH 7.4.
 3. Trypsin (Difco 1 : 250), in Hanks' lacking NaHCO_3 , at a concentration of 0.25 per cent.
 4. Sera, human, horse and sheep, inactivated at 56° C for 30 minutes.
 5. Lactalbumin hydrolysate, 5 per cent solution prepared in NaHCO_3 -free Hanks' solution.
 6. Trypan blue solution: the supernatant of a 2 per cent solution of crystalline trypan blue centrifuged at 452 g for 10 minutes.
 7. Culture media: 73 per cent Hanks', 5 per cent lactalbumine hydrolysate, 20 per cent serum, 2 per cent NaHCO_3 —carbonic acid buffer.
 8. Maintenance solution: 87.5 per cent Hanks', 2.5 per cent lactalbumine hydrolysate, 5 per cent NaHCO_3 —carbonic acid buffer, 5 per cent serum.
 9. Glutamine solution containing 2.92 mg/ml glutamine in distilled water.
 10. Mycostatin Squibb: 500 000 U, dissolved in 20 ml ethylene glycol.
- The culture media contained 100 IU/ml penicillin and 100 IU/ml streptomycin.

Cleaning of glassware. After sterilization the glassware was washed in tap water with mechanical cleaning. This was followed by boiling in a 1 per cent solution of lauryl sulphonate for 30 minutes and repeated washing in tap water. The cleaning procedure was finished by

boiling in 1 per cent versene (disodium EDTA) for 15 minutes and repeated washing in distilled water.

The rubber stoppers were treated according to WELLER's method [5].

Digestion of amniotic membranes with trypsin. The afterbirths were collected in a sterile beaker and the membrane cut off with scissors and put in a 500 ml flask containing 200 ml of Hanks' solution. Care was taken to avoid the contact of the membrane with antiseptic substances. After delivering the membrane to the laboratory, the amnion was separated from the chorion with forceps, cut into pieces of 3 cm by 3 cm and washed repeatedly in Hanks' solution. The cells from the amniotic membrane cleared from blood clots were obtained by trypsin treatment. This was carried out either at 33° C or at 37° C. Preliminary trypsinization lasted 30 minutes independently of the temperature. This first fluid containing mostly cell debris was discarded and the amnion pieces were washed three times in Hanks'. Trypsinization at 33° C was carried out in a water bath for 5 to 6 hours, if the membrane had been obtained during the day, and overnight, for 12 to 14 hours, when it had been brought in the evening. If trypsinization lasted only 6 hours, the flask was shaken at 30 r. p. m. and an amplitude of 5 cm. Membranes to be trypsinized at 37° C were kept in an incubator for 3 hours.

Preparation of cell suspension. After trypsinization the flask was carefully shaken to release the cells still attached to the membrane. The cell suspension obtained was passed through a gauze filtre into a centrifuge tube. The membranes were rinsed with Hanks' to release further cells, and the suspension thus obtained was filtered into the centrifuge tube. Cells were sedimented at 1000 r. p. m. for 10 minutes and washed twice in Hanks' by centrifugation at 800 r. p. m. The packed cells were suspended in 5 ml Hanks' solution by means of a capillary pipette and the cell number was determined in a Bürker chamber.

Staining of cells with trypan blue. 0.5 ml of the trypan blue solution was added to 1 ml of the cell suspension and stirred. The cells were examined immediately after staining, establishing the ratio of stained to unstained cells in a Bürker chamber at a magnification of 80×.

Preparation of culture tubes. The cell suspension was diluted to contain 300 000 cells per ml and each of the culture tubes was seeded with 1 ml of this suspension. The rubber stoppered culture tubes were incubated at 36° C in a slanted position.

Results

In our experiments an effort has been made both to obtain by trypsinization as many viable cells as possible and to maintain the cultures in a viable condition and with intact morphology for at least two weeks, since they were to be used for cultivation of human pathogenic viruses. Moreover our aim was to be informed immediately after trypsinization about the fitness of the cells to yield tissue cultures. This was judged from the morphology and trypan blue staining of the cells. The usefulness of all the methods mentioned above was controlled on the basis of the cells' ability to settle and to form a confluent sheet. Cells failing to settle within 72 hours or not forming a confluent sheet within 8 to 10 days were considered unsuitable for tissue culture. The morphology of the cell cultures was examined unstained at a magnification of 80×. Only cultures containing epithelial-like, vacuole-free cells, with clear cytoplasm and intact nucleus were regarded as suitable, if their morphology remained intact for at least two weeks.

In the course of the studies we examined first, whether temperature and duration of the trypsinization had an influence on cell yield and viability. The pertaining data are presented in *Table I*.

The amnion cells obtained at the two different temperatures proved equally suitable for culturing since at 33° C 60 per cent and at 37° C 65.5 per

Table I

Comparison of results of trypsinization at 33° and 37° C

Temperature	Number of amnions	For culture preparation				Average yield in million
		suitable		unsuitable		
		number	per cent	number	per cent	
33°	121	73	60	48	40	65.3
37°	131	86	66	45	34	84.8
Total	252	159	63	93	37	

cent of the amnions yielded adequate cells. As it made no difference in yield whether trypsinization at 33° C lasted 5 or 12 hours, these observations have not been detailed in *Table I*.

Though the cells of the different amnion specimens immediately after trypsinization showed a great variety in morphology, they could be divided into 3 groups: (I) regular, roundish, cyst-like, transparent, clear cells with no granules; (II) irregular and moderately granulated cells; (III) dark and copiously granulated cells. Cells belonging to the first group were found to be most suitable for tissue culture. They quickly settled and a confluent cell sheet was readily formed. Those belonging to the second group showed a variable cultivability. Some of these cell suspensions settled, though relatively slowly, and a confluent sheet was only formed after repeated changes of the culture medium. Cells of most of the amnion membranes examined belonged to this group. Cells belonging to the third group were not suitable for cultivation. Nevertheless, morphology did not appear to be a good basis for judging the viability of the cells obtained by trypsinization as only the first and third group offered a possibility for inferring as to their suitability. Most of the amnions yielded cells of intermediary morphology, *i. e.* they belonged to the second group characterized by unpredictable viability.

The harvest of the different trypsinized amnions was variable. Cells of the amnion membranes giving a better harvest proved to be more suitable for preparing cultures inasmuch as 66 per cent of the membranes yielding more than 70 million cells could be used for cultivation, while only 49 per cent of those yielding less than 70 million. As neither cell morphology nor the yield were a sufficient basis for judging the suitability of the cells for preparing cultures, the reliability of the trypan blue method used among others by BECKER [6] was also tested. According to BECKER's opinion, if more than 10 per cent of the cells were stained, the suspension was not suitable for tissue culture. Trypan blue staining was applied by us to 156 amnion membranes. Data of these examinations are to be found in *Table II*.

Table II

*Comparative examination of the suitability of cells for tissue cultures
and of the trypan blue staining*

The ratio of staining	Number of amnions	For culture preparation			
		suitable		unsuitable	
		number	per cent	number	per cent
under 10 per cent	114	86	75	28	25
above 10 per cent	42	17	40	25	59

According to our data, cells of which more than 10 per cent are stained by trypan blue may also be suitable for tissue culture, while those of which less than 10 per cent took the stain did not always prove suitable.

Since neither cell morphology, nor the yield or the trypan blue staining proved a wholly reliable characteristic of the cells' condition, some preliminary culture tubes were set up to check viability, while the bulk of the cells were stored in 100 ml flasks with 20 ml culture medium containing 10 per cent glutamine. The flasks, containing 20 million cells each, were kept at room temperature for 24 to 48 hours. After storage the cell number was again determined, as meanwhile part of the cells adhered to the flasks bottom. Similarly to the fresh ones, of those stored in tubes 300 000 cells were used for tissue cultures. Apparently no change of viability occurred during storage.

The culture medium was first completed with horse serum, but recently, on DUNNEBACKE's [7] recommendation, we have used a culture medium completed with sheep serum. This was found to be the most advantageous. To elucidate possible differences between sheep and human sera in their influence on tissue cultures, a preliminary experiment was set up with culture media containing sheep resp. human serum. The data for this experiment are summarized in *Table III*.

Table III

Suitability of the sera employed for culture media

Sera	Number of amnions	For culture preparation			
		suitable		unsuitable	
		number	per cent	number	per cent
Horse	90	45	50	45	50
Sheep*	141	88	62	53	38
Human*	141	91	65	50	35

* Evaluated on the basis of 10-10 preliminary tube cultures.

Our data, in agreement with DUNNEBACKE's, showed both sheep and human serum to be equally superior to horse serum.

Discussion

The preparation of a primary amnion tissue culture involves some particular difficulties. Proper collection of the amnions and their delivery to the laboratory is often cumbersome. A further disadvantage is that not every amnion membrane yields suitable cells, so the time consuming trypsinization in these cases is fruitless. For this reason the use of established amnion strains seems advantageous. There are, however, circumstances when preference must be given to primary amnion cultures, the established strain being less susceptible to ECHO viruses [8]. We also had to use primary amnion cultures and this is what has made us to try to diminish the difficulties associated with their production.

For trypsinization we chose the temperature of 37° C instead of 33° C because the length of time needed is shorter and the yield somewhat greater.

So far no method has been developed by which to judge the amnion membranes whether or not their cells will be suitable for preparing tissue cultures. Neither is there an adequate method for checking the viability of cells after trypsinization, since cell morphology, the yield and trypan blue staining are often unreliable. To avoid the eventually superfluous task of working up all cells to tissue cultures, preliminary culture tubes were used. Until their evaluation (24 to 48 hours) the bulk of the cells can be stored without damage.

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Address of the authors:

ILONA BÉLÁDI, ESZTER KUKÁN, IMRE MÉCS, ERVIN SZÖLLÖSY

Institute for Microbiology, University Medical School, Beloiannisz tér 10, Szeged, Hungary.

AGAR DIFFUSION TEST AND MICROMETHODS FOR THE RAPID BIOCHEMICAL DIFFERENTIATION OF ENTERIC BACTERIA

By

B. LÁNYI and MARIA M. ÁDÁM

State Institute of Hygiene, Budapest

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Summary. A method has been developed for the rapid and simple identification of enteric bacteria. The colony to be examined is inoculated into a combined urease-indole medium. After some hours of incubation the culture is streaked on the surface of a desoxycholate-phenol red agar plate. Acid production from glucose, mannitol, lactose, inositol, sucrose and dulcitol is determined on the latter medium by use of dry paper slips impregnated with these substances. The same plate serves for the demonstration of gas and hydrogen sulphide production and for the determination of the methyl red reaction. Desoxycholate-phenol red agar effectively suppresses the growth of contaminating organisms. A simple spot test has been developed for the tryptophan deaminase reaction.

In a comparison based on the examination of 479 strains the results obtained by the new method and by the standard tests agreed in nearly 100 per cent. A somewhat greater difference (6.7 per cent) was shown by the methyl red test. The practical value of the method has been confirmed by the routine examination of non-faecal specimens. With the 12 rapid reactions the systematic position of 95.2 per cent of 649 freshly isolated Enterobacteriaceae strains could be determined.

The modern classification of the family Enterobacteriaceae is far from making easier the work of the medical bacteriologist. The proper identification of enteric bacteria has become definitely more difficult, since for the grouping of these organisms numerous and often time-consuming biochemical tests are required. The widely-used simple conventional methods give a more or less sufficient information only when salmonellae or shigellae are concerned. Among other enteric bacteria occurring in faecal samples the more common members of the former groups can quickly and easily be selected on the basis of their characteristic growth on selective and differential media and by use of well-elaborated serological procedures. The accurate routine identification of other enteric bacteria presents considerable difficulties, although the exact determination of these would often be desirable. For example, it is known that under normal conditions urine, bile, sputum and other non-faecal materials contain no enteric bacteria. When these are isolated from such specimens, it is customary to report their name together with their antibiotic sensitivity. By most bacteriologists instead of the more general terms "enteric bacterium" or "organism belonging to Enterobacteriaceae", a name restricted to a definite group of bacteria (*e. g.* *E. coli*, *Klebsiella*) is given. These workers neglect the fact that the small number of biochemical reactions usually performed do not justify the use of such nomenclature. It is no wonder that wide variations can

be found in the statistics of various laboratories as to the occurrence of different groups of Enterobacteriaceae.

The members of the family Enterobacteriaceae are divided into groups on the basis of a series of biochemical reactions. It stands to reason that in routine examinations such a large number of reactions, many of which requires a long incubation time, cannot be practically performed even in well-equipped large laboratories. For smaller laboratories the very preparation and storage of the numerous differential media mean a considerable difficulty. The troublesome registration of large numbers of media in various stages of incubation is another disadvantage. On the other hand, it is clear that the acceleration of the reports is a matter of great importance. Thus, if we insist on giving a definite name to the isolated organism without loss of time, we have to choose biochemical reactions suitable for the characterization of Enterobacteriaceae within a short incubation time.

Many simple and quick biochemical reactions have been recommended. The rapid methods for the demonstration of urease production have proved satisfactory for practical purposes [1–11]. Culture media have been devised for showing urease production in combination with other tests [12–20]. A quick and reliable result is given by the amino acid deaminase reactions [21, 22]. The simplified amino acid decarboxylase tests [23] as well as the accelerated methods for the demonstration of gelatin hydrolysis are similarly well-suited for practical purposes [24–28].

A number of rapid microreactions for fermentation [29–38], H_2S production [39, 40], nitrate reduction [41, 42], citrate utilization [43], indole production [44, 45, 46], methyl red [47] and Voges—Proskauer reaction [48] have been worked out. A series of microreactions were described by CLARKE and COWAN [49]. These fermentation and other microreactions, although they give results within a few hours, have several disadvantages. Often their results do not correspond to those obtained by the standard tests or are disturbed by chemical impurities or bacterial contamination. The most important disadvantage of these procedures is that performing them is more than labour-consuming. Thus they are hardly fit for laboratories carrying out large-scale diagnostic work.

In order to simplify the fermentation and other reactions several authors impregnated the substrates into paper discs and paper strips or incorporated them in tablets [50–57]. The advantages especially for smaller laboratories of a set of storable differential media are obvious. Of these methods the most developed and less complicated are those in which the paper discs are placed on the surface of an inoculated agar plate.

The agar diffusion method for the examination of the acid production by enteric bacteria was first applied by KNOX [50]. SNYDER [55] impregnated paper discs with solutions containing all the usual constituents necessary for

the different biochemical reactions. SANDERS, FABER and COOK [57] described a medium containing phenol red and ammonium ferrous sulphate, from which, similarly to SNYDER's method, pour plates were prepared by inoculation of the culture into the melted medium. The dried paper discs containing only the fermentable substances were placed on the surface of the plate.

The preparation of pour plates required by both of the latter methods allows the detection of gas formation and shortens the incubation time to not more than 6 hours. The reduction of incubation time may be important when a rapid identification of a suspected *Salmonella* or *Shigella* strain is necessary. Practically, however, it is clear that the preparation of pour plates means a considerable task in laboratories examining large numbers of specimens. Reactions giving a result within hours are expressly unnecessary for the grouping of enteric bacteria isolated from nonfaecal materials, as in that case prior to reporting the result the antibiotic sensitivity of the pathogenic agent ought also to be determined. The generally-used antibiotic disc method, as known, requires incubation of the plates overnight.

In the present work, taking into consideration the methods of other authors, rapid and simple tests have been developed which allow the grouping of pathogenic or facultative pathogenic enteric bacteria with an accuracy satisfactory for practical purposes.

Materials and methods

Test cultures. The organisms used were partly freshly isolated, partly obtained from the culture collection of this laboratory. Part of the *Escherichia* strains were kindly supplied by Dr. MARGARET GREGÁCS, *State Institute of Hygiene, Budapest.*

Comparison of biochemical reactions. The rapid tests were compared with standard biochemical procedures described in the *International Bulletin of Bacteriological Nomenclature and Taxonomy* [58]. If the standard reactions of strains obtained from the culture collection had been determined in earlier examinations, the control tests were not performed.

Culture media. 1. *Combined liquid medium for the detection of urease and indole production.*

Distilled water, 900 ml; Bacto peptone (Difco), 10 g; NaCl, 2 g; Phenol red, 0.2% solution, 6 ml. The solution is distributed at 180 ml portions into flasks and sterilized in the steamer. To 180 ml basal medium the following Seitz-filtered solution is added. Distilled water, 17 ml; KH_2PO_4 , 10% solution, 1 ml; K_2HPO_4 , 10% solution, 2 ml; Urea, 2 g. Finally the medium is distributed in 2 to 3 ml amounts into small sterile test tubes. The combined medium is inoculated with a loopful of culture. Readings can be made after 16 to 22 hours of incubation at 37° C.

Urease reaction: ++ Before the addition of the indole reagent the medium is red. The reagent produces gas (CO_2) formation in the medium and white fumes (NH_4Cl) over the surface of the liquid.

+ Before the addition of the indole reagent the medium is red. The reagent produces gas and fumes slightly or not at all.

— Before the addition of the indole reagent the medium is of yellow or orange colour.

Indole reaction. Indole reagent according to Kovács [59]: 5 g p-dimethylamidobenzaldehyde dissolved in 75 ml iso-amylalcohol. To 3 parts of the solution 1 part concentrated HCl is added (v/v). After the addition of the HCl the mixture should be used only for a limited time. To the cultures approximately 0.2 ml indole reagent is pipetted and the tubes are slightly shaken.

+ The overlaying reagent is dark red.

The overlaying reagent is yellow.

2. *Desoxycholate-phenol red (D. P.) agar for the demonstration of fermentation and H₂S production.*

Basal medium: Tap water, 1000 ml; Agar, 18 g; Beef extract, 10 g; Peptone (Richter), 10 g; NaCl, 5 g; Na₂S₂O₃ · 5 H₂O, 1 g; KH₂PO₄, 1 g; K₂HPO₄, 3 g; N NaOH, 15 ml; Sodium desoxycholate, 2 g; Phenol red 0.2% solution, 10 ml; Fluorescein 0.5% solution, 4 ml.

Instead of sodium desoxycholate 2 g desoxycholic acid may be used. Before addition to the medium the latter should be dissolved by heating in the necessary quantity of N NaOH. The reaction of the basal medium is adjusted so as to correspond to pH 7.2 after the addition of iron ammonium citrate. After the basal medium is clarified by sedimentation, it is distributed at 1000 ml portions into flasks and sterilized in the autoclave. The basal medium is storable practically for an unlimited time.

Before use the basal medium is melted in the steamer and to 1000 ml of the hot agar 2.5 g iron ammonium citrate ("green scales") dissolved in 25 ml distilled water is added. Before addition the solution is brought to a boil. Into each Petri dish (diameter 90 mm) about 25 ml medium should be poured. The plates can be stored in the refrigerator for 1 or 2 weeks.

Preparation of 0.2 per cent phenol red solution. Phenol red, 1 g; 0.1 N NaOH, 40 ml; distilled water 460 ml.

Preparation of paper slips containing the fermentable substances. The slips with glucose were prepared as follows. In 6.5 ml distilled water 3.5 g glucose was dissolved, then the solution was brought to a boil. After cooling to 50° C, 15 × 200 mm sterilized Macherey-Nagel No. 214 filter paper strips were dipped into it. The strips were dried on a wire net for some hours at 75° C then at room temperature. Finally they were sheared into pieces of 15 × 15 mm in size. Each paper slip prepared this way contained approximately 24 mg glucose.

Paper slips containing the other fermentable substances were prepared by a different procedure. Filter paper sheets of 90 × 190 mm size were marked with coloured wax pencils by drawing 6 lines parallel to the long side of the sheet. The papers for the various fermentable substances were signed with different colours. Each of the sheets was sterilized, then wetted and laid in a 90 × 190 mm glass container in which washed mercury had previously been placed. The mercury formed a continuous layer on the bottom of the container. Over the wet paper sheet laid on the surface of the mercury, 30 ml of "agar base" containing the required fermentable substance was poured. The "agar base" consisted of distilled water 165 ml, agar 5.5 g, gelatin 3 g. To a 30 ml portion of the autoclaved "agar base" 4 g fermentable substance was added and the mixture was kept in a 100° C water bath for 2 to 3 minutes. The hot mixture was then poured on the paper sheet. After the agar layer had solidified the sheet was pulled off the surface of the mercury, placed on a wire-net and dried at room temperature. Next day the sheet was cut into 15 × 15 mm pieces each marked by a coloured bisecting line. Thus from one sheet 72 paper slips were prepared, each containing 50 mg of the fermentable substances. The completely dried slips were stored in cardboard boxes. During storage some of the materials crystallized on the surface. The crumbling away of the substances was prevented by the gelatin incorporated in the "agar base". For the preparation of the paper slips sterile conditions were not necessary. The sterilization of the paper sheets and the "agar base" was carried out chiefly from mere habit.

Examination of fermentation and H₂S production on D. P. agar. The plates were seeded with bacteria grown in liquid medium by means of glass rods. Then the paper slips were placed on the surface of the plates. The film serving as an indicator of gas production was likewise affixed to the medium (Fig. 1). The media were incubated overnight at 37° C.

Fermentation: + Yellow, opaque zone around the corresponding paper slip.

— The medium remains unchanged.

H₂S production: + Black band at the periphery of the acid zones or blackening of the medium under the film,

— The medium remains unchanged.

Gas production. For trapping the gas produced from glucose pieces of used X-ray film were used. The emulsion from a sheet of 35 × 35 cm film was washed off in warm water with a brush, then both of its surfaces were covered with a layer of egg white. The white of one egg was mixed with 10 ml water and one half part of this mixture was spread on one side of the film. After it had been dried at room temperature, the other side of the film was similarly treated. When the film was completely dry, it was cut into squares of 20 × 20 mm.

For trapping the gas one piece of film was placed onto the surface of a plate, close to the slip containing glucose. After incubation the gas production was shown by bubbles and by a more or less fissuring of the agar under the film.

Methyl red reaction. After an overnight incubation of the D. P. agar plates onto the paper slip containing glucose one small drop of 0.25 per cent alcoholic methyl red (Merck) solution was pipetted.

- + Bright red colour
- Light yellow colour.

Tryptophan deaminase spot test. Plastic plates described by MANDULA [60] were used. Each of these were supplied with 20 circular hollows 13 mm in diameter. Into each of the hollows one drop of saturated tryptophan solution was pipetted. Then a loopful of a culture grown on D. P. agar plates was suspended in the solution. Care was taken to use bacteria grown on parts of the medium showing no acid reaction. Thus on one plastic plate altogether 20 different strains could be examined. After suspending the bacteria the plate was covered with another plastic plate. Metal buttons protruding about 2 mm from the surface of the plates prevented the airtight covering of the hollows. The plates were incubated for 1 hour at 37° C then to each suspension 1 drop of ferric chloride reagent was added.

- + Cherry-red coloration.
- Own colour of the reagent (light yellow).

Saturated tryptophan solution: 0.1 g DL-tryptophan dissolved by heating in 10 ml distilled water. After cooling, 0.5 ml 0.2 per cent merthiolate is added.

Ferric chloride reagent: 5 g FeCl₃ dissolved in 100 ml 0.01 N HCl.

Scheme of identification of bacteria by the rapid method. One isolated colony of the organism to be examined is transferred into the combined urease-indole medium. From this culture after 3 to 6 hours of incubation first the nutrient agar plates serving for the estimation of antibiotic sensitivity, then one D. P. agar plate are seeded by means of a glass rod or a cotton swab. The culture is dispersed evenly on the surface of the plates. Then the paper slips containing the fermentable substances are affixed to the D. P. plate. The film is placed close to the glucose paper. The plates are put in the incubator and the combined urease-indole medium is also incubated. The biochemical reactions including the indole and methyl red tests are read next morning. If necessary for the identification of the strain, the tryptophan reaction is also performed.

Results

Combined medium for the demonstration of urease activity and indole production. For demonstrating the decomposition of urea and the production of indole in one and the same medium various methods have been elaborated [61–67]. Our own medium is a modification of SZITA's peptone water [15] which has been found very useful in routine work. The results given by the combined medium are presented in *Table I*. It is seen that within 22 hours only *Proteus* bacteria gave a strong urease reaction (red colour and intensive development of NH₄Cl fumes and CO₂ gas after the addition of the indole reagent). The medium was reddened by part of the *Klebsiella* and by some *Cloaca* strains; in the tubes with these, however, fumes or gas were produced slightly or not at all. In the control urea medium after 24 hours out of 47 *Klebsiella* strains only 22, out of 58 *Cloaca* strains only 3 were positive. In addition to the mentioned bacteria, 2 strains showed a late decomposition of urea. These, on the basis of other reactions, were included in the *Escherichia* group. Cultures of enteric bacteria not decomposing urea were yellow or orange in the combined medium. When shigellae were cultured the medium nearly always remained yellow. Most of the *S. typhi* and *Escherichia* strains exhibited a similar behaviour. Other enteric bacteria caused an orange colour.

On longer incubation further changes occurred in the colour of the combined medium. With the majority of *Klebsiella* and *Cloaca* strains it became red on the second day. A similar change was produced by *Salmonella*, *Arizona*, *Citrobacter* and *Providencia* strains on the third or fourth day. After 4 days

Table I

Comparison of biochemical reactions in combined urease-indole medium and in standard media
Total number of strains 479

	No. of strains	Urease		Indole	
		Combined medium*	Control**	Combined medium*	Control***
Salmonella	65	65 —	65 —	65 —	65 —
Arizona	15	15 —	15 —	15 —	15 —
Citrobacter	37	37 —	37 —	37 —	37 —
Klebsiella	47	15 + 32 —	41 + 6 —	6 + 41 —	6 + 41 —
Cloaca	58	3 + 55 —	50 + 8 —	58 —	58 —
Escherichia	80	80 —	2 + 78 —	73 + 7 —	75 + 5 —
Shigella	48	48 —	48 —	19 + 29 —	19 + 29 —
<i>P. vulgaris</i>	33	33 +++	33 +	33 +	33 +
<i>P. mirabilis</i>	30	28 ++ 1 + 1 —	29 + 1 —	30 —	30 —
<i>P.morganii</i>	28	28 ++	28 +	28 +	28 +
<i>P. rettgeri</i>	9	9 ++	9 +	9 +	3 + 6 —
Providencia	14	14 —	14 —	5 + 9 —	7 + 7 —
<i>Ps. pyocyanea</i>	15	15 —	15 —	15 —	15 —

* Combined medium 37° C 22 hrs.

** Kristensen's fluid medium 37° C, 4 days

*** 1% peptone water 37° C 4 days, Kovács's reagent

Escherichia strains caused partly a red, partly an orange colour; the combined medium remained yellow only with shigellae and some *S. typhi* strains. After 4 days the indole reagent caused a strong NH_4Cl and CO_2 reaction in cultures of *Proteus* strains. At the same time *Klebsiella* and *Cloaca* cultures were characterized by a weak NH_4Cl and an occasional, uncertain CO_2 reaction. Among other enteric bacteria the indole reagent produced after 4 days a minimal quantity of white fumes; development of CO_2 was never observed.

The behaviour of urease negative cultures in the combined medium is evidently due to a production from the peptone of a quantity of alkali sufficient to change the pH of the buffered medium on longer incubation. Urease media of the buffered system contain generally less peptone (0.1 per cent) than other media. Our combined medium in order to yield a good growth and indole production requires at least 1 per cent peptone. After 2 or 3 days of incubation the medium is no longer able to buffer the alkali formed from the peptone. The control medium, CHRISTENSEN's agar modified by KRISTENSEN [68], gave a distinct colour reaction after 4 days, as in that case the alkali was neutralized by the acid supplied by the fermentation of glucose. In the combined medium, which served also for the indole test, evidently no glucose could be incorporated.

As shown in *Table I*, indole production in the combined medium after 22 hours almost completely corresponded to that in the standard medium after 4 days. Some late indole producing strains occurred only in the *Escherichia* and *Providencia* groups. It is interesting that the majority of *P. rettgeri* strains gave a negative indole reaction after 4 days in peptone water. In reality all of the latter strains produced indole, as similarly to the result obtained in the combined medium, in 22 hour old peptone water cultures they were all indole positive. According to the literature, the *Providencia* group is indole positive.

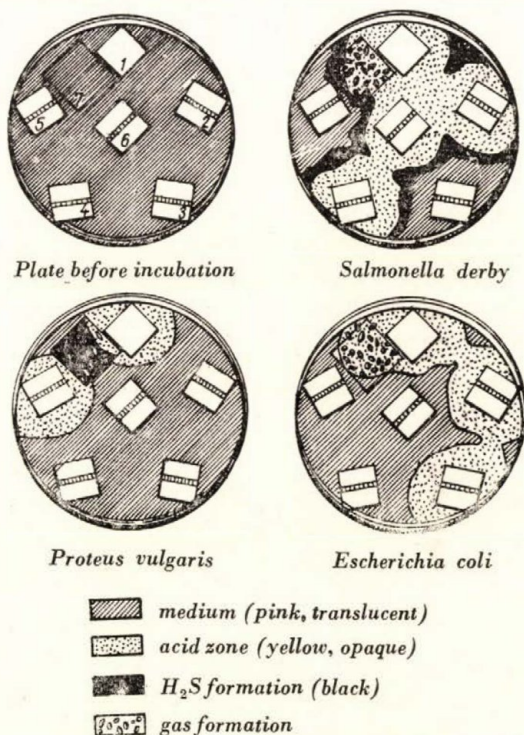


Fig. 1. Characteristic reactions of enteric bacteria on desoxycholate-phenol red agar
1 = glucose (white), 2 = mannitol (red), 3 = lactose (brown), 4 = inositol (green), 5 = sucrose (yellow), 6 = dulcitol (blue), 7 = film

Yet, the indole negative strains presented in *Table I* were, on the basis of other biochemical reactions, included in this group.

Desoxycholate-phenol red (D. P.) agar for testing fermentation and H_2S production. *Table II* shows the growth of various microorganisms on D. P. agar. The high selectivity of the medium is evident. A luxuriant growth was exclusively shown by bacteria belonging to Enterobacteriaceae and Pseudomonadaceae. Yeast-like organisms isolated from the faeces of infants grew fairly well on D. P. agar. These, however, similarly to the two strains isolated from the air, were able to multiply on desoxycholate citrate agar as well.

Among Enterobacteriaceae, some strains belonging to the groups *Klebsiella*, *Cloaca* and *Escherichia* were more or less inhibited by the medium. Serotypes believed to be more sensitive than others, as *S. abortus equi*, *S. typhi suis*, *S. typhi*, *S. sendai*, *S. pullorum*, *Sh. dysenteriae* 1 grew abundantly after 18 hours. As the medium contained thiosulphate, the "dwarf" form of *S. typhi* also grew well. Due to the presence of desoxycholate, *Proteus* strains did not swarm on this medium. Organisms occurring in the air which might be taken into consideration as contaminative agents were practically completely inhibited. When stored for a longer period in the refrigerator or at room temperature, mainly colonies of molds appeared on the plates.

Fermentation tests on D. P. agar. During incubation a change was observed in the original colour of the surface-streaked D. P. plates. Due to alkalinization the faint pink, fluorescent medium became deeper in colour. (The fluorescein content of the medium served for the differentiation of D. P. agar

Table II
Growth of various microorganisms on D. P. agar
Total number of strains, 582

Organism	No. of strains	Growth
<i>Salmonella</i>	65	64 +++++, 1 ++++
<i>Arizona</i>	15	13 +++++, 1 +++++, 1 ++
<i>Citrobacter</i>	37	35 +++++, 2 +++++
<i>Klebsiella</i>	50	43 +++++, 3 +++++, 1 ++, 1 +, 2 -
<i>Cloaca</i>	59	43 +++++, 10 +++++, 4 ++, 2 -
<i>Serratia</i> and <i>Hafnia</i>	3	3 +++++
<i>Escherichia</i>	80	65 +++++, 8 +++++, 7 ++
<i>Shigella</i>	48	48 +++++
<i>Proteus vulgaris</i>	33	32 +++++, 1 ++++
<i>Proteus mirabilis</i>	30	30 +++++
<i>Proteus morgani</i>	28	22 +++++, 6 ++++
<i>Proteus rettgeri</i>	9	9 +++++
<i>Providencia</i>	14	14 +++++
<i>Pseudomonas pyocyanea</i>	15	15 +++++
Other <i>Pseudomonas</i> strains	15	11 +++++, 4 ++++
Yeast-like organisms	10	10 ++
Molds (<i>Penicillium</i>)	10	10 ×
<i>Streptococcus faecalis</i>	10	9 ×, 1 -
<i>Staphylococcus aureus</i>	10	10 -
<i>Staphylococcus albus</i>	6	1 ×, 5 -
Aerobic spore-forming bacilli	10	10 -
Bacteria isolated from the air	25	1 +++++, 1 +++++, 2 ×, 21 -

+++++ Luxuriant growth after 18 hrs.

++++, ++ More or less inhibited growth; biochemical reactions distinguishable after 18 hrs.

+ Strongly inhibited growth; biochemical reactions not distinguishable after 18 hrs.

× Late and poor growth

- No growth within 6 days

from other similarly coloured media.) The medium was the least rendered alkaline by *Shigella* and *Escherichia* strains. The acid zones developing during 18 hours' incubation were approximately circular, bright yellow, opaque areas 20 to 35 mm in diameter (*Fig. 1*). The size of the zones varied with the bacteria tested. The largest zones were caused by salmonellae, the smallest by the methyl red negative bacteria. When the plates were further incubated, the acid zones disappeared and the whole medium became alkaline. Complete reversal of the reaction did not occur before 48 hours. When salmonellae were cultivated, the reversal took place only after a longer period. With methyl red negative bacteria it could be distinctly observed after 48 hours. Delayed fermentation was also reliably demonstrated on D. P. agar. Late lactose-fermenting *E. coli* and *Sh. sonnei* strains did not produce acid from lactose within the short period of incubation. After some days a well-defined although less intense acid zone developed around the paper containing lactose. The late reaction, similarly to the early one, disappeared after one or two days. *Ps. pyocyanea* strains formed mucoid colonies, difficult to remove; as an indication of weak acid production the medium became yellow in colour around the glucose paper generally without precipitation of desoxycholic acid.

Desoxycholate, apart from making the medium highly selective, has the advantage of lending much contrast to the acid zones of the medium. In acid medium desoxycholate is precipitated and the resulting fine desoxycholic acid precipitate together with the adsorbed indicator forms bright yellow, opaque zones. When beef extract was omitted from D. P. agar, the acid zones were even more distinct and the reversal of the reaction ensued considerably later. The medium devoid of beef extract, however, inhibited the growth of a large number of *P.morganii*, *Klebsiella* and *Cloaca* strains.

The results of the fermentation reactions obtained by the diffusion technique and by the standard method are compared in *Tables III/a* and *III/b*. For the sake of reality, the 18 hour reading of the control tubes is presented. It is seen that the two methods agree completely only as regards the fermentation of glucose. As to other substances, acid production occurred often earlier with the diffusion method than with the control. *E.g.* the fermentation of mannitol by 1 *P. rettgeri* strain, of lactose by 3 Arizona strains, of inositol by 5 *Cloaca* and 4 *P. rettgeri* strains, of sucrose by 4 *Escherichia* and 4 *P. mirabilis* strains and 1 *Providencia* strain was observed on D. P. agar after 18 hours, when the corresponding liquid media still showed a negative reaction. The alternative of this (negative by the diffusion and positive by the control test after 18 hours) occurred altogether with 9 strains.

Gas production. The gas formed from glucose was demonstrated by Knox's method [47]. Instead of the expensive cover glasses pieces of X-ray film were employed. As shown in *Table III/b*, with 4 exceptions the result given by bacteria producing a large amount of gas were similar to that ob-

Table III/a
Comparison of biochemical reactions on *D. P.* agar and in standard media
Total number of strains, 479

	No. of strains	Glucose		Mannitol		Lactose		Inositol	
		D. P.	Control	D. P.	Control	D. P.	Control	D. P.	Control
<i>S. typhi</i>	15	15 +	15 +	15 +	15 +	15 -	15 -	15 -	15 -
<i>S. paratyphi A</i> , etc.*	9	9 +	9 +	8 + 1 -	8 + 1 -	9 -	9 -	9 -	9 -
Other salmonellae	41	41 +	41 +	41 +	41 +	41 -	41 -	18 + 23 -	21 + 20 -
Arizona	15	15 +	15 +	15 +	15 +	3 + 12 -	15 -	15 -	15 -
Citrobacter	37	37 +	37 +	37 +	37 +	20 + 17 -	20 + 17 -	37 -	37 -
Klebsiella	47	47 +	47 +	46 + 1 -	47 +	41 + 6 -	41 + 6 -	43 + 4 -	44 + 3 -
Cloaca	58	58 +	58 +	58 +	58 +	51 + 7 -	51 + 7 -	10 + 48 -	5 + 53 -
Escherichia	80	80 +	80 +	80 +	80 +	69 + 11 -	69 + 11 -	1 + 79 -	1 + 79 -
<i>Sh. dysenteriae</i> 1-7	11	11 +	11 +	11 -	11 -	11 -	11 -	11 -	11 -
<i>Sh. flexneri</i> , <i>boydii</i>	22	22 +	22 +	19 + 3 -	19 + 3 -	22 -	22 -	22 -	22 -
<i>Sh. sonnei</i>	15	15 +	15 +	15 +	15 +	15 -	15 -	15 -	15 -
<i>P. vulgaris</i>	33	33 +	33 +	33 -	33 -	33 -	33 -	33 -	33 -
<i>P. mirabilis</i>	30	30 +	30 +	30 -	30 -	30 -	30 -	30 -	30 -
<i>P. morganii</i>	28	28 +	28 +	28 -	28 -	28 -	28 -	28 -	28 -
<i>P. rettgeri</i>	9	9 +	9 +	9 +	8 + 1 -	9 -	9 -	9 +	5 + 4 -
Providencia	14	14 +	14 +	14 -	14 -	14 -	14 -	6 + 8 -	6 + 8 -
<i>Ps. pyocyanea</i>	15	15 +	15 +	15 -	15 -	15 -	15 -	15 -	15 -

* *S. abortus equi*, *typhi suis*, *sendai*, *pullorum*

D. P. = desoxycholate-phenol red agar 37° C 18 hrs

Control = standard medium 37° C 18 hrs.

Table III/b

Comparison of biochemical reactions on *D. P.* agar and in standard media

Total number of strains 479

	No. of strains	Sucrose		Dulcitol		Gas production		H ₂ S production	
		D. P.	Control	D. P.	Control	D. P.	Control	D. P.	Control
<i>S. typhi</i>	15	15 —	15 —	15 —	15 —	15 —	15 —	15 —	15 +
<i>S. paratyphi A</i> , etc.*	9	9 —	9 —	2 + 7 —	2 + 7 —	8 + 1 —	8 + 1 —	2 + 7 —	2 + 7 —
Other salmonellae	41	41 —	41 —	33 + 8 —	34 + 7 —	40 + 1 —	40 + 1 —	41 +	41 +
Arizona	15	15 —	15 —	15 —	15 —	13 + 2 —	15 +	13 + 2 —	15 +
Citrobacter	37	11 + 26 —	11 + 26 —	24 + 13 —	24 + 13 —	37 +	37 +	37 +	37 +
<i>Klebsiella</i>	47	42 + 5 —	43 + 4 —	12 + 35 —	13 + 34 —	47 +	47 +	47 —	47 —
Cloaca	58	57 + 1 —	58 +	58 —	58 —	58 +	58 +	58 —	58 —
<i>Escherichia</i>	80	31 + 49 —	27 + 53 —	40 + 40 —	40 + 40 —	78 + 2 —	80 +	80 —	80 —
<i>Sh. dysenteriae</i> 1—7	11	11 —	11 —	11 —	11 —	11 —	11 —	11 —	11 —
<i>Sh. flexneri, boydii</i>	22	22 —	22 —	22 —	22 —	1 + 21 —	1 + 21 —	22 —	22 —
<i>Sh. sonnei</i>	15	15 —	15 —	15 —	15 —	15 —	15 —	15 —	15 —
<i>P. vulgaris</i>	33	33 +	33 +	33 —	33 —	29 + 4 —	33 +	33 +	33 +
<i>P. mirabilis</i>	30	4 + 26 —	30 —	30 —	30 —	29 + 1 —	30 +	30 +	30 +
<i>P.morganii</i>	28	28 —	28 —	28 —	28 —	28 +	28 +	28 —	28 —
<i>P. rettgeri</i>	9	9 —	9 —	9 —	9 —	9 —	9 —	9 —	9 —
Providencia	14	1 + 13 —	14 —	14 —	14 —	14 —	3 + 11 —	14 —	14 —
<i>Ps. pyocyanea</i>	15	15 —	15 —	15 —	15 —	15 —	15 —	15 —	15 —

* *S. abortus equi*, *typhi suis*, *sendai*, *pullorum*

D. P. = desoxycholate-phenol red agar 37° C 18 hrs.

Control = standard medium 37° C 18 hrs.

tained by the control examination. Differences were more frequent with the less intensively gas producing *Proteus* and related bacteria. It should be noted that the blackening under the film caused by *P. vulgaris* and *P. mirabilis* often hindered the recognition of gas formation.

H₂S production. The results are shown in the last column of Table III/b. It was striking that in contrast to the control, on D. P. agar every *S. typhi* strain should give a negative H₂S reaction. Apart from these and 2 Arizona strains, the results agreed with the control. As in the medium of SANDERS *et al.*, the H₂S production by *Salmonella*, Arizona and *Citrobacter* appeared in the form of 5 mm wide black rings at the periphery of the acid zones. Unlike in the medium of the former authors, on D. P. agar *P. vulgaris* and *P. mirabilis* caused blackening only under the film and under the paper slips containing substances not attacked by these bacteria. A negative reaction was given by *P. morganii*, corresponding to the opinion of RAUSS and VÖRÖS [69], who stated that in thiosulphate medium this organism did not produce H₂S.

The difference in the appearance of H₂S reaction between D. P. agar and the medium of SANDERS *et al.* may be due partly to various ingredients of the media. As regards D. P. agar, for the clear cut indication of H₂S production the proper balance between phosphate and iron is essential. A decrease in the amount of the former and an increase of the latter made the H₂S production by *Proteus* more demonstrative. Formation of H₂S is highly-dependent on the pH of the medium. The more acid the reaction, the less intense the blackening. With respect to other biochemical tests and the selectivity of the medium, of the numerous variations tried the described formula gave the best results. In D. P. agar the source of sulphur was the thiosulphate; a medium devoid of this compound gave no positive H₂S reaction.

The black band formation in pour plates at the edge of the acid zone was explained by COOK and PELCZAR [70] as follows. Fermentation brings about reduced conditions and decreases the high oxygen tension inhibiting the production of H₂S. Thus H₂S is produced only in the acid zone. Here, however, its compound formed with the iron indicator is unstable. Accordingly, FeS accumulates only at the edge of the zone, where the pH is sufficiently high. As to the surface-streaked D. P. plate, particularly with *P. vulgaris* and *mirabilis*, the conditions for H₂S formation were presumably less favourable. Under the film and the paper slips, probably owing to an effective reduction of the oxygen tension during growth, these bacteria were also able to cause an accumulation of FeS.

Methyl red reaction. In the course of the present work the most considerable difficulties were met with when the development of a workable M. R. microtest was attempted. It is known that M. R. positive and negative bacteria can be reliably differentiated only in a medium containing peptone, glucose and phosphate buffer in a certain constant proportion. According to

COWAN [47], in the early stages of growth both positive and negative bacteria produce a pH that lies in the critical range of the methyl red indicator. On further incubation the M. R. positive organisms increase the acid production, while the M. R. negative ones break down the acids and cause a reversion towards neutrality. Accordingly, the outcome of the M. R. reaction depends on the duration of incubation as well as on the constituents of the medium. This explains the observation that M. R. negative organisms, unlike the others, produce a small fermentation zone on D. P. agar and the reversal of the zone is comparatively rapid.

The first part of Table IV compares the results obtained by the M. R. microtest and by the standard M. R. reaction. Divergent results were obtained in 32 out of 479 examinations (6.7 per cent). The highest differences were found in the *P. rettgeri* and Providencia groups. It should be noted that these bacteria are known to give the standard M. R. test often with a doubtful positivity.

Table IV

Comparison of methyl red and tryptophan deaminase microreactions and the standard tests
Total number of strains, 479

	No. of strains	M. R. microtest	M. R. standard test*	Tryptophan spot test	Phenylalanine test**
Salmonella	65	59 + 6 -	65 +	65 -	65 -
Arizona	15	13 + 2 -	15 +	15 -	15 -
Citrobacter	37	37 +	37 +	37 -	37 -
Klebsiella	47	2 + 45 -	4 + 43 -	47 -	47 -
Cloaca	58	6 + 52 -	1 + 57 -	58 -	58 -
Escherichia	80	70 + 10 -	75 + 5 -	80 -	80 -
Shigella	48	48 +	48 +	48 -	48 -
<i>P. vulgaris</i>	33	33 +	33 +	33 +	33 +
<i>P. mirabilis</i>	30	30 +	30 +	30 +	30 +
<i>P. morgani</i>	28	26 + 2 -	28 +	28 +	28 +
<i>P. rettgeri</i>	9	5 + 4 -	9 +	9 +	9 +
Providencia	14	10 + 4 -	14 +	14 +	14 +
<i>Ps. pyocyanea</i>	15	2 + 13 -	15 -	15 -	15 -

* Standard methyl red medium 37° C 4 days

** Henriksen's test

The M. R. test is a sensitive part of the present method. The use of peptone or meat extract other than indicated or a shift in the initial pH of the medium may make the results unreliable. When new constituents are introduced or when a large series of D. P. agar is prepared, the M. R. reaction should be titrated with positive and negative strains. In case of D. P. agar

the M. R. reaction chiefly depends on the proper concentration of glucose. Thus for the titration paper slips should be impregnated with solutions containing various amounts of glucose.

Tryptophan deaminase spot test. The test is a modification of the method described by SINGER and VOLCANI[22]. According to the present examinations, the shaking of the cultures in test tubes can be replaced by an incubation of the suspensions in wide and flat hollows of a plastic plate. The suspensions should be readily accessible to the air.

A positive reaction is given within 45 to 60 minutes by enteric bacteria capable of producing indole-3-pyruvic acid by the oxidative deamination of tryptophan. The former compound gives a cherry-red colour reaction with ferric ions. The spot test is much simpler to carry out than the other amino acid deaminase reactions. As the results are in perfect agreement with HENRIKSEN's phenylalanine reaction [21], the test is most suitable for the rapid differentiation of the *Proteus-Providencia* groups and other enteric bacteria (*Table IV*).

Discussion

As shown by the Tables the 12 different biochemical reactions described sufficiently characterize the various Enterobacteriaceae groups for practical purposes. However, the exact identification of certain groups may require additional tests.

For the routine differentiation of *Salmonella*, *Arizona* and *Citrobacter* strains in addition to the rapid tests the amino acid decarboxylase, the KCN and the quick gelatin reactions may be useful. The identification of prompt lactose-splitting *Escherichia* strains is not difficult. The late lactose-fermenting members of this group can be recognized without delay on the basis of their other reactions. Since there is no final agreement in the literature as to the characteristic biochemical behaviour of *Klebsiella* and *Cloaca*, in routine examinations no attempts have been made to distinguish between members of these groups. For the differentiation of *Proteus* from other Enterobacteriaceae, there is usually no need to perform the tryptophan test, while if a strain is suspected to belong to *Providencia*, the test must be carried out.

The purpose of the present work was the development of a simple method which might help bacteriologists working in smaller laboratories. The D. P. agar offers the great advantage of rendering the sterile technique unnecessary. Unlike media stored in tubes, non-selective agar plates are particularly liable to contamination and provide an ample chance of obtaining false reactions. When D. P. plates are used, the cultures should be free only from other enteric bacteria. In contrast to other similar techniques, the present method does not require the time-consuming preparation of pour plates.

Some months ago the method has been introduced in this laboratory for the routine identification of enteric bacteria isolated from non-faecal materials. Since that time 649 Enterobacteriaceae strains were cultured. The distribution of the strains was as follows. *Salmonella* 2, *Citrobacter* 21, *Klebsiella-Cloaca* 178, *Escherichia* 227, *Proteus-Providencia* 190. The position of 31 strains was doubtful. Accordingly, 95.2 per cent of the strains could be identified by means of the 12 rapid reactions.

The experience gained on routine material thus showed that the method makes possible a practically accurate identification of enteric bacteria without any loss of time. The routine examinations clearly showed that the basic requirement of the method is the inoculation of isolated colonies. In spite of the greatest care, the cultures were sometimes vitiated by other enteric bacteria. Another considerable advantage of D. P. plates over the liquid media was that such mixed cultures were easy to recognize. Obviously, the method is adequate for the biochemical confirmation of the serological identification of salmonellae and shigellae.

Finally, it should be noted that the agar layer technique used for the preparation of the paper slips is not regarded as a necessary part of the method. For the large scale manufacture of these, the substances may be compressed into tablets. The impregnation of very thick blotting paper sheets may also be useful, provided that the crystallization and crumbling of certain substances can be prevented.

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Address of the authors:

BÉLA LÁNYI, MARIA M. ÁDÁM,

National Institute of Hygiene, Gyáli út 2-6, Budapest IX, Hungary.

POLIOMYELITIS PROPHYLAXIS IN HUNGARY

By

T. BAKÁCS

State Institute of Hygiene, Budapest

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Summary. Epidemiological observations together with serological data revealed 70 to 80 per cent protection within 1 year following Salk vaccination with three doses. The protection began to decrease in the second year after the vaccination. The disease contracted by vaccinated persons usually took a mild course. Salk vaccination did not interfere with the enteric multiplication of polio virus, thus circulation of the virus among the population was not inhibited.

As the Sabin type vaccine seems to be lacking the disadvantages of the Salk vaccine and has proved superior to the latter in many additional respects, Hungarian health authorities have decided to use only the Sabin vaccine in the future.

In Hungary the first experimental vaccination with live polio vaccine was performed in the autumn of 1959. The vaccine was prepared from SABIN's original attenuated strains by M. P. CHUMAKOV (Moscow) [1, 11]. The total child population of one county, 100 000 children ranging in age from 3 months to 14 years were given the trivalent experimental oral vaccine. The vaccination was started on November 2, 1959 and completed on November 7. A second dose of trivalent vaccine was given to the same group in December.

A country-wide vaccination with monovalent live polio vaccine was started 5 weeks after the experimental vaccination with the trivalent preparation. The monovalent vaccines were applied in intervals of 4 to 5 weeks in the sequence of types 1, 3 and 2. The vaccinated age group was the same as with the experimental vaccination. A total of round 2 200 000 children received the monovalent vaccines.

Regular, compulsory Salk vaccinations having been carried out in Hungary since 1957, one might ask for the reasons of changing the apparently successful vaccine. We shall now discuss these reasons on the basis of the results obtained at the different departments of the *State Institute of Hygiene, Budapest*.

In 1957 a severe epidemic of poliomyelitis was observed in Hungary [8, 10]. While the incidence of cases was still increasing in July 1957, the Health Authorities organized a compulsory Salk vaccination programme resulting in the vaccination of 1 200 000 children, mostly belonging to the age group between 0 to 3 years. The increasing tendency of the morbidity curve was changed soon after the vaccination and the epidemic appeared to be con-

trolled about October, 1957. [9]. The year 1958 was non-epidemic, while in 1959 another wave of poliomyelitis invaded the country [13, 14].

In the following the epidemiological evaluation of the Salk vaccination campaign performed during the epidemic in 1957 will be given together with an analysis of the possible factors involved in the 1959 epidemic.

A total of 2334 poliomyelitis cases was recorded during the 1957 epidemic. The morbidity curve had an atypical shape, having been favourably influenced by the mass vaccinations. By means of the probability theory it was possible to calculate the theoretical shape of the morbidity curve if no vaccination would have been made. The figures of calculated and those of actually found values are presented in *Table I*.

Table I

*Poliomyelitis cases in Hungary in the period from 1952 to 1956 and in the year 1957.
Computed number of cases for August to December, 1957,
as calculated from the average seasonality in the period from 1952 to 1956*

Period	Total from 1952 to 1956		1957		1957	
	Number of cases observed				Number of cases computed	
	No.	per cent	No.	per cent	No.	per cent
January to July	1164	31.4	1411	60.5	1411	31.4
August to December	2542	68.6	923	39.5	3083	68.5
Total	3706	100.0	2334	100.0	4494	100.0
<i>Monthly distribution</i>						
January	74	2.0	59	2.5	59	1.3
February	40	1.1	52	2.2	52	1.2
March	54	1.4	39	1.7	39	0.9
April	63	1.7	50	2.1	50	1.1
May	125	3.4	145	6.2	145	3.2
June	257	6.9	361	15.5	361	8.0
July	551	14.9	705	30.2	705	15.7
August	717	19.3	487	20.9	869	19.3
September	758	20.5	265	11.4	919	20.5
October	549	14.8	97	4.2	666	14.8
November	334	9.0	45	1.9	404	9.0
December	184	5.0	29	1.2	225	5.0
Total	3706	100.0	2334	100.0	4494	100.0

The same values as plotted in a graph are presented in *Fig. 1*.

Neither *Table I* nor *Fig. 1* leave any doubt about the effectiveness of the performed Salk vaccination and about 2000 persons seem to have been protected by the application of the preventive measure.

In the light of these earlier results and the fact that from 1957 to 1959, 70 to 90 per cent of the population under 20 years of age had been vaccinated with Salk vaccine, it was difficult to explain how the 1959 epidemic had come about. One of the most probable explanations seemed to be the decrease of immunity. Though Salk himself supposed his vaccine to produce protection for 3 to 4 years [12], a remarkably shorter duration was generally experienced. The 1959 epidemic in Hungary seems to be one of the data supporting this general observation.

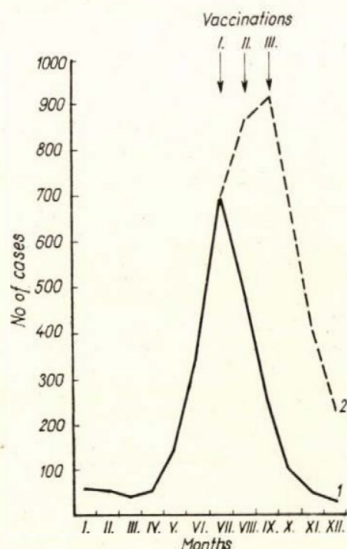


Fig. 1. Monthly distribution of poliomyelitis cases in Hungary in 1957

Explanations:

1. Number of cases actually recorded
2. Theoretical number of cases as calculated on the basis of data from the period 1952 to 1956

A total of 1830 poliomyelitis cases were recorded in 1959. The detailed history and epidemiology of these cases revealed the protection induced by the preceding Salk vaccination to be still present, but exhibiting a decreasing tendency.

Table II

Distribution of cases in 1959 according to previous Salk vaccinations

Number of cases recorded	No. 1769	Per cent 100.0
Written record of 3 or more vaccinations	627	35.4
Claimed to have been vaccinated	48	2.7
Total	675	38.1

Table II presents the vaccination history of the poliomyelitis patients from the 1959 epidemic. Only 35.4 per cent of those contracting poliomyelitis in 1959 had a written record of vaccination with 3 doses and an additional 2.7 per cent certainly remembered to have been subjected to vaccination.

The Salk vaccinations carried out since 1957 had a remarkable influence on the distribution of morbidity in the most susceptible age groups. The relative incidence of poliomyelitis in 1957 was the highest in the age group of 1 to 2 years, while in 1959 in the children between 3 to 9 or more years of age. This shift might have been due to the fact that infants under 1 year of age did not have natural or artificial immunity, while children who in 1959 were 1 to 2 years old were vaccinated in the same or in the previous year so that their artificial immunity must have been about its peak at the time of the epidemic. In the older children immunity due to the 1957 vaccination was mostly declining. This diminished immunity might explain the comparatively high incidence of the disease in these age groups in 1959. This conclusion had suggested the health authorities to make facilities for revaccination in the winter and June 1959. Revaccination was performed with 1 ml single doses. As a result of extensive Salk vaccinations in 1957 and partly of the revaccinations in 1959 about 1500 persons seemed to have been protected in the 1959 epidemic.

Table III
Incidence of poliomyelitis in different age groups

Age group	1957	1959
0 years	14.2 per cent	20.9 per cent
1 to 2 years	47.2 „ „	30.5 „ „
3 to 9 years	28.0 „ „	37.3 „ „
over 10 years	10.6 „ „	11.3 „ „
Total	100.0 „ „	100.0 „ „

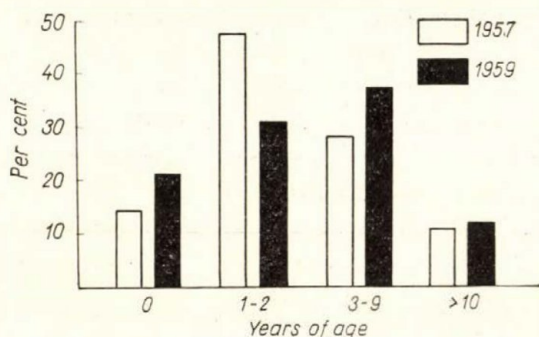


Fig. 2. Incidence of poliomyelitis in different age groups in the years 1957 and 1959

To give a general scope about the poliomyelitis incidence in Hungary during the period 1954 to 1959, the morbidity per months is presented in Fig. 3.

The remarkably low incidence of poliomyelitis in 1958 must mostly have been resulted from the extensive Salk vaccination carried out in 1957. One should not disregard the natural immunizing effect of the very severe 1957, epidemic. Moreover, the widespread Bornholm epidemic in 1958 might also

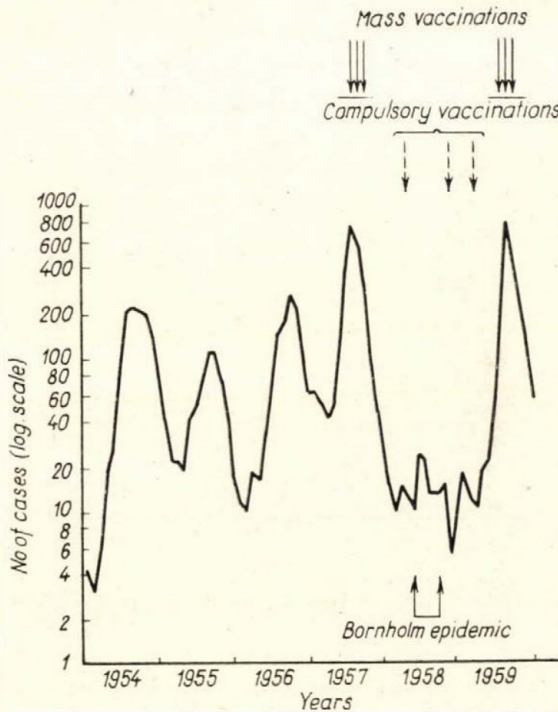


Fig. 3. Poliomyelitis morbidity per month in Hungary in the period from 1954 to 1959

have controlled by interference the free circulation of poliomyelitis virus in the population [2-6]. The Bornholm epidemic caused by Coxsackie B3 virus [2] was the most severe in the Transdanubian area.

As to the role of interference in controlling the circulation of polio virus we refer to a study performed in a children's collective [7]. In this case ECHO 7 virus infection occurred among type 1 polio virus excretors; the result was a rapid elimination of the latter organism from the intestines, parallel with the appearance of the new agent. These events are graphically presented in Fig. 4.

Both Figs. 3 and 4 give some further support for assuming a number of factors other than serological immunity in influencing the epidemiological, pathological, and clinical events in poliomyelitis.

There are still some obscure points in the pathomechanism of poliomyelitis. In contrast to the earlier supposition concerning the obligatory neurotropism of the virus, the new the primary site of invasion is thought to be the intestinal tract, especially its part richly provided with Peyer-plaques. As to the further ways of spreading in the organism different theories have been suggested, each of them being supported by a number of different data. Opinions mostly agree in one point, the utmost importance of virulence in spreading whatever its ways may be. The possible role of the tonsils and the other parts of the pharynx is still under discussion, but it has been proven that in some cases the virus is harboured in the throat, thus making droplet infection possible. The intestinal tract, however, is the most important site, as it does

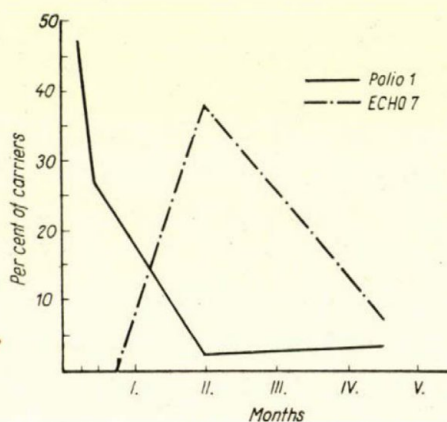


Fig. 4. Interference between type 1 polio and type 7 ECHO viruses

not only harbour the virus, but also makes possible its extensive multiplication and excretion in large amounts. Thus poliomyelitis is at present looked upon as an enteral infection, having all the epidemiological characteristics of the enteric diseases.

Epidemiologically, great importance attaches to the fact that the central nervous system becomes involved in only one of 100 or 1000 cases, according to the data of the different authors. This means that the infection is predominantly latent, producing a long lasting natural immunity of the child population. Naturally as sources of infection, the latent carriers are a great danger and they may serve as centres of an epidemic outbreak. Poor hygienic conditions, great traffic, overcrowded housing provide favourable conditions for the spreading of the virus and thus for latent natural immunization. In backward countries poliomyelitis virus contaminates children in their very early age, often in that sheltered by maternal immunity. As poliomyelitis virus is characterized by high virulence, its circulation cannot be inhibited even by

high standards of hygiene, thus children will mostly contract infection before the age of 14 years even in countries with excellent social and hygienic conditions.

The characteristics and the ways of spreading of polio virus might serve to explain the facts that, in spite of its high virulence paralytic cases are few and that the sequence of epidemiological events very often appears to be in-continuous.

Table IV

*Spreading of type 1 polio virus in children's collective
two weeks after the first observed paralytic case**

Designation	Number examined	Excretor	
		No.	per cent
Children 0 to 3 years of age	80	50	62.5
Nursing staff	54	4	7.4

* An accidental ECHO 7 virus infection interfered with further observation

Table IV presents the spreading of type 1 poliovirus within a children's collective. Two weeks after the occurrence of three manifest cases of poliomyelitis 62.5 per cent of the healthy contacts excreted type 1 polio virus. This was remarkable as immediately after the appearance of the first cases the most rigorous epidemiological measures were introduced.

Table V

Virus excretion of previously seronegative and seropositive persons

	Number of children examined	Number excretors at		
		first	second	Total
		sampling		
Seropositive	19	6	2	8
Seronegative	22	12	3	15
Total	41	18	5	23

In Table V the data for excretion are presented in relation to the presence of antibodies produced by previous Salk vaccination [7]. It is apparent that humoral antibodies, though somewhat reducing the period of excretion, were unable to interfere with the multiplication of virus in the intestinal tract. The few data obtained for the nursing staff were in agreement with some earlier statements and the low incidence of excretors was certainly a result of their high natural immunity.

Discussion

In agreement with the literary data available, the Salk vaccine proved to be an effective prophylactic against poliomyelitis in our hands. Its effectiveness was found to be 70 to 80 per cent for about a year's period after the administration of a complete series of 3 times 1 ml. On the basis of the epidemiological data collected in Hungary in the years 1957 and 1959, we suggest revaccination every two years for communities still making use of the killed, Salk-type vaccine. Revaccinations should preferably be carried out before the epidemic season.

The 20—30 per cent of appropriately vaccinated persons were still found to have remained susceptible though if they had contracted poliomyelitis the disease usually took a mild form (moderate, mostly transitory paralysis) and fatalities were rare.

Even appropriately performed mass Salk vaccination did not seem to have eliminated the "wild" virus from the population and high antibody titres in the serum were not found to interfere with virus multiplication in the intestinal tract. Thus apparently Salk vaccination is inadequate for a radical elimination of eventual sources of epidemics.

The impressive amount of recently-accumulated favourable foreign data on the Sabin vaccine and our own not quite satisfactory experience with the Salk vaccine has led us to give preference to the Sabin vaccine and since December, 1959, this preparation has served for the compulsory vaccinations in Hungary.

Acknowledgement. The author is greatly indebted to G. BARSY for supplying the statistical data.

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Address of the author:

TIBOR BAKÁCS

State Institute of Hygiene, Gyáli út 2—6, Budapest IX, Hungary.

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AN ELECTRON MICROSCOPIC STUDY OF MYCOBACTERIUM PHAGES

By

F. GUBA and EDITH VANDRA

Department for Micromorphology of the Research Institute for Technical Physics of the Hungarian Academy of Sciences, Budapest and Diagnostic Laboratory of the National Institute for Tuberculosis "Korányi", Budapest

(Received January 12, 1960)

Summary. The morphological characteristics of five Mycobacteriophage species (*M. friburgensis*, *M. phlei*, *M. rabinowitsch*, *M. smegmatis*, *M. butyricum*) were studied by electron microscopy. The phages are tadpole-like and differ in the head's diameter and the tail's length.

Since the first isolation of Mycobacteriophages 14 years ago most of the reports on this subject have been concerned with the demonstration of phages in connection with some phenomena observed, and not with the phages themselves [1—5]. PENSO, who has been the most successful in isolating Mycobacteriophages, published a detailed electron microscopic study and described the morphological characteristics of these phages [6]. He also presented quantitative data concerning the growth cycle of some of his phages completed by electron microscopic studies [7, 8].

In a previous paper [9] we reported on the isolation of phages each capable of lysing one or more of seven saprophytic Mycobacteria. In this report we present electron microscopic investigations showing that our phages are morphologically different from each other.

Materials and methods

Bacteria and phages. The following species of Mycobacteria and the homologous phages were investigated: *M. friburgensis*, *M. rabinowitsch*, *M. phlei*, *M. smegmatis* and *M. butyricum*. The bacterial strains were kindly supplied by the International Strain Collection, Lausanne; the phages were isolated by us. Mycobacteria were cultivated in 5 ml 2 per cent glycerol broth for 48 hours. The cultures were then homogenized by shaking with stainless small-shot. As diluent the culture medium was used.

Tenfold dilution series were prepared from the stock solutions of phages. Twenty ml 2 per cent agar medium were poured into Petri dishes and kept at 37° C for 48 hours. The sterile plates were then overlaid by the phage-bacterium-agar mixture. To prepare this mixture, 0.9 ml of varying dilutions of the phage suspension mixed with 0.1 ml bacterial suspension was added to 3 ml 1 per cent agar medium containing 1 per cent glycerol. The medium was cooled to 45° C before mixed. This method is the same as that described by HERSHEY *et al.* [10] except for the agar and glycerol contents of the medium.

The plaques became countable by the 24th hour of the incubation at 37° C.

Preparation for electron microscopy. At the beginning of this work the drop preparation method was used. The phage as suspended in broth was dropped on a collodion membrane, the preparation was washed and shadowed with palladium. The results were not satisfactory. Washing with distilled water did not free the preparation of some disturbing materials deriving from the broth. So we changed over to the so-called replica method. Plates with a few separate

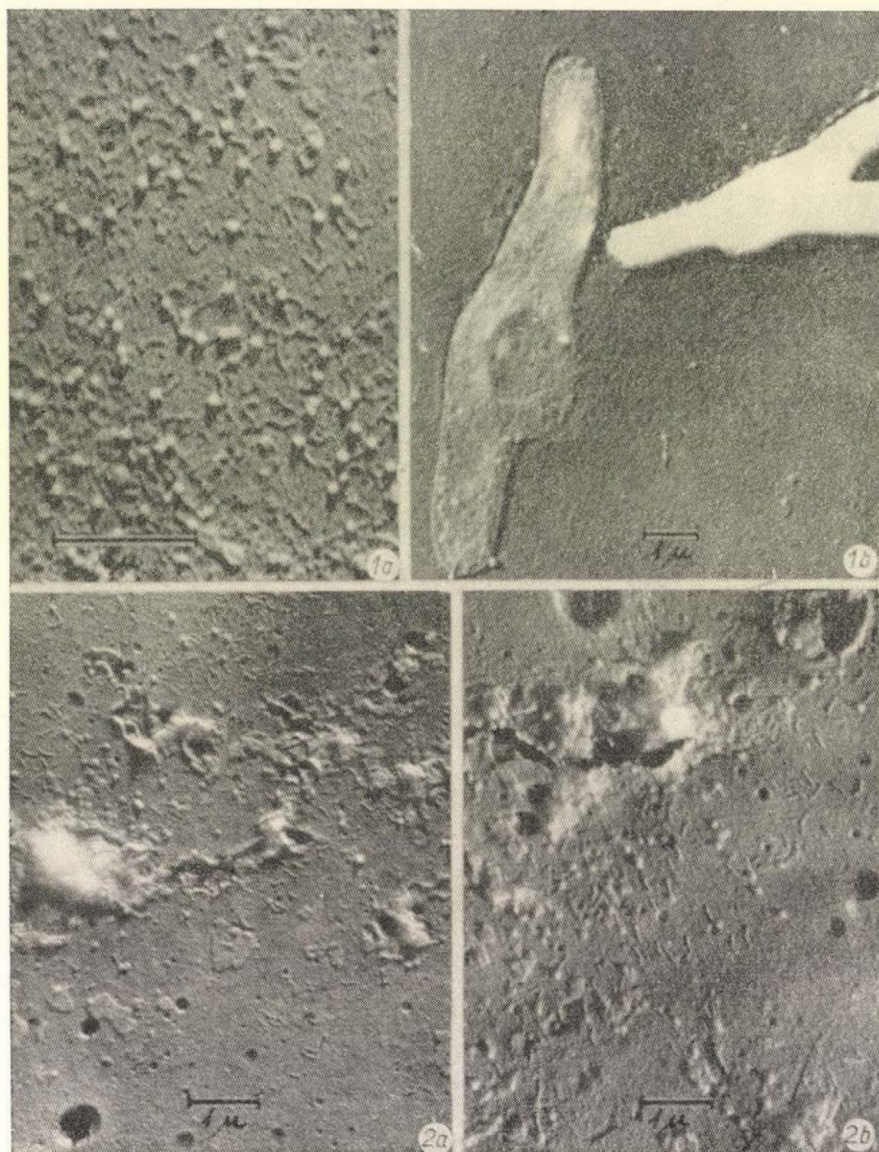


Fig. 1a and b. Phagus friburgensis

Fig. 2a and b. Phagus phlei

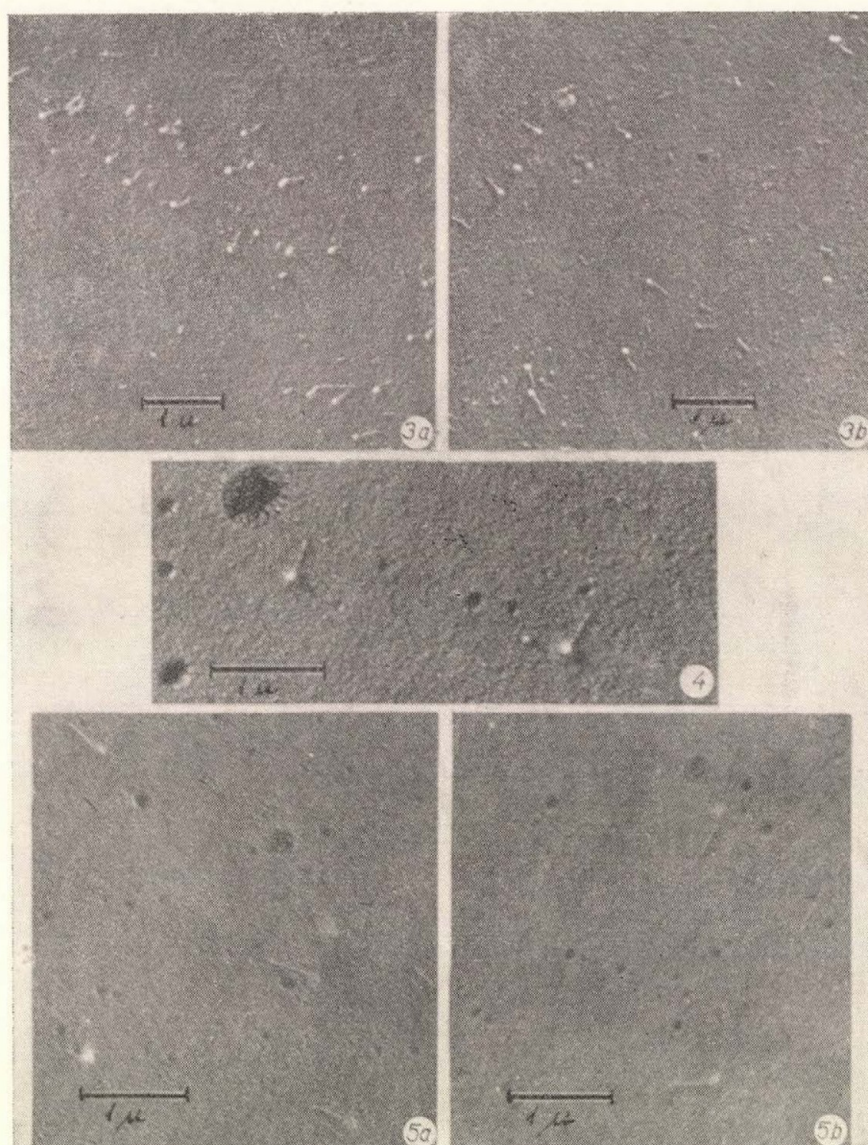


Fig. 3a and b. Phagus rabinowitsch
Fig. 4. Phagus smegmatis
Fig. 5a and b. Phagus butyricus

plaques were covered with 0.2 to 0.5 per cent collodion in amyl acetate, the amyl acetate was allowed to evaporate, and the agar medium was cut into small pieces at the site of each plaque. The collodion layer on the top of the agar pieces was allowed to float on the surface of distilled water, and upside down, taken out by a micro screen. In this manner pseudoreplica of phages and bacteria were obtained [11]. The preparations were shadowed with palladium.

Experimental

The Mycobacteriophages isolated by us are tadpole-shaped, as shown in *Figs. 1 to 5*. They have an electrodense head and a tail. We have measured the head's diameter and the tail's length for each of the five phage species. The head's diameter was found to vary between 100 m μ and 140 m μ , the tail's length between 200 m μ and 400 m μ . Thus, the latter was highly variable by species. The data are summarized in *Table I*.

Table I

Average diameter of heads and length of tails of five Mycobacteriophage species

Phage	Head m μ	Tail m μ
Phagus friburgensis	100	200
Phagus phlei	120	210
Phagus rabinowitsch	100	230
Phagus smegmatis	100	280
Phagus butyricus	140	400

Standard deviation was generally about 10 per cent

Table I shows that the five Mycobacteriophages are different in size. PENSO found the head of his Mycobacteriophages double-granulated. We did not find double granulation.

Acknowledgement. The authors thank Miss CLARA SZIVESSY for the electron microscopic examinations.

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Address of the authors:

FERENC GUBA

Department for Micromorphology of the Research Institute for Technical Physics of the Hungarian Academy of Sciences, Puskin u. 11, Budapest VIII, Hungary,

EDIT VANDRA

The Diagnostic Laboratory of the National Institute for Tuberculosis "Korányi", Pihenő ut 1, Budapest, XII, Hungary.

STUDIES ON THE INCIDENCE OF *E. COLI* SEROTYPE O : 125 B : 15 (VAR. CANIONI)

By

G. UJVÁRY and G. PÁLL

Paediatric Department of the 14th District Town Council Hospital, Budapest

(Received February 2, 1960)

Summary. During a hospital epidemic the rare enteropathogenic strain *E. coli* O : 125 B : 15 was isolated in 52 cases out of faecal samples collected from 1033 sick infants and children. The morphology, biochemical activity, serology and antibiotic sensitivity of the strains isolated was examined. Out of the 52 patients 30 had enteral symptoms. Symptoms were mild in 18, medium in 9 and severe in 3 cases. Twenty-two patients had no enteral symptoms. Attention is drawn to the importance of the resistance being decreased by extraenteral pathologic processes, particularly influenza and its complications. The therapy applied and the environmental factors possibly playing a role in the spreading and development of the epidemic are discussed. On the basis of the findings we regard type O : 125 B : 15 of *E. coli* as a facultative enteral pathogenic agent in infants.

TAYLOR and CHARTER [1] were the first to isolate *E. coli* var. *Canioni* from faecal samples of children suffering from gastroenteritis. In further studies the strain proved to be of serotype O:125 B:15 and its possible role in infantile dyspepsia was suggested.

At our laboratory *E. coli* O:125 B:15 was first isolated in January, 1959, from routine faecal samples. As data concerning the incidence and the pathologic role of this strain were scarce in the literature, it was decided to carry out a detailed study. In the present paper the results of our morphological, biochemical and serological investigations will be reported, and the clinical forms and epidemiology of the disease caused by *E. coli* O:125 B:15 will be discussed.

Since its first isolation in January, 1959, the routine faecal bacteriology of infants and children was made with special regard to the strain during a 6 months period. Independently of the nature of their actual disease, 1033 infants and children belonging to the age group from 0 to 4 years were examined. In 52 cases we succeeded in isolating *E. coli* O:125 B:15. The isolations were successful in most cases also on repeated attempts.

Materials and methods

For the bacteriological examination of faecal samples the common agar-plate media (blood agar, eosin-methylene-blue, desoxycholate, bismuthsulphite) were used, and a polytropic medium instead of Russel's medium. Serological identification and typing of the strains isolated from the faeces of infants and children with enterocolitis was made by "OB" rabbit immune sera obtained by immunization with standard *E. coli* strains of type B1 to B16.

As a result of our systematic serological analyses we had succeeded in isolating the rare *E. coli* strain B : 13 from a case of infantile enterocolitis [2].

The colony morphology of the recently isolated O : 125 B : 15 type strain could best be observed on eosin-methylene-blue agar plates. The colonies were characterized by a smooth dull surface, crenated edge and a central bluish grey coloured button. Thus they could be tentatively differentiated from other *E. coli* strains by mere inspection. A preliminary identification of the strains was made by slide agglutination using "OB" serum in 1 to 20 dilution and a cell suspension obtained from 24 hour old eosin-methylene-blue agar plate cultures. Strains giving typical B agglutination with O : 125 B : 15 serum were further examined by the tube agglutination method, using "OB" serum dilutions from 1 to 50 to 1 to 6400. The cell suspensions used for agglutination were obtained from 24 hour old agar slant cultures and used partly untreated, partly after 2 and a half hours of boiling in saline. As a control, untreated and similarly-treated suspensions of the standard strain were used.

Results

Bacteriological observations. In Table I the results of agglutination experiments obtained with the 52 isolated strains are presented according to the serum titres together with the results obtained with sera of rabbits immunized with types I and II of the O:125 B:15 strains isolated.

Table I
Results of agglutination tests

Sera obtained by immunization with	Strains					
	Standard O : 125 B : 15		Isolated type I		Isolated type II	
	living	boiled	living	boiled	living	boiled
Standard O : 125 B : 15	1 : 400	1 : 3.200	1 : 400	1 : 3.200	1 : 3.200 ±	1 : 3.200
Isolated O : 125 B : 15	1 : 800	1 : 6.400	1 : 800	1 : 12.800	1 : 12.800	1 : 12.800

As revealed in Table I, the strains were agglutinated by specific serum exactly to the same titre as the standard O:125 B:15 strain. The strains designated as type I and giving a typical "B" agglutination when untreated and a typical "O" agglutination when boiled, amounted to 42. The other 10 strains designated as type II gave a mixed "B—O" type agglutination when untreated, and had an "O" agglutination titre similar to that given by the standard strain. This was regarded a sign of the variation of "O" inagglutinability of the surface "B" antigen. The agglutination titres of sera prepared by immunization with the isolated strains contained both "O" (O:125) and "B" (B:15) antibodies.

As a final proof of the identity of the isolated *E. coli* strains and the O:125 B:15 standard strain, the cross antibody absorption reaction was also performed. The 10 strains designated by us as type II also possessed the B:15

antigen which, however, was demonstrable only by the absorptive potency because of the lack of its "O" agglutination-inhibiting ability. *Table II* presents the results of the cross-absorption tests.

Table II
Results of absorption tests

Sera obtained by immunization with	Absorbed with strain	Agglutination reaction with strains					
		Standard O:125 B:15		Isolated type I		Isolated type II	
		living	boiled	living	boiled	living	boiled
Standard	Isolated type I	neg.	neg.	neg.	neg.	neg.	neg.
O: 125 B: 15	Isolated type II	neg.	neg.	neg.	neg.	neg.	neg.
Isolated O: 125 B: 15	Standard O: 125 B: 15	neg.	neg.	neg.	neg.	neg.	neg.

The standard O:125 B:15 strain of our laboratory was a ciliated variant characterized also by H 19 antigen. An unciliated variant of the same strain is, however, well-known from the description of TAYLOR and CHARTER [1]. Because of the lack of appropriate specific anti-"H" immune sera we could only demonstrate the active movement of our strains making use of the U-tube technique. All the 52 strains isolated in our laboratory were found to be ciliated variants.

Carbohydrate fermentation and biochemical activity of all of our O:125 B:15 strains were found to agree with the characteristics as described by KAUFFMANN [3] for *E. coli*. All of our strains fermented adonite. This characteristic, however, is only seldom present in other *E. coli* strains. In *Table III* are seen the results of fermentation and biochemical tests of our strains.

The antibiotic sensitivity of the strains was examined on blood agar plates using "biotest" filtre paper discs (prepared by the Institute for Sero-bacteriological Production and Research "Human", Budapest). Similarly to their antigenic structure, fermentation and biochemical characteristics, the antibiotic sensitivity of our 52 *E. coli* O:125 B:15 strains was also uniform. The strains were sensitive to streptomycin, chloramphenicol, terramycin and neomycin, while all were resistant or only slightly sensitive to sulphonamides, penicillin, aureomycin, erythromycin and polymixin B. Quantitative testing of the streptomycin, chloramphenicol, terramycin and neomycin sensitivity was performed on ten O:125 B:15 strains selected at random. The experiment was made in tubes containing 2 ml broth and 100 µg, 50 µg, 25 µg, 12,5 µg, 6,25 µg, 3,12 µg, 1,56 µg and 0,78 µg each of the antibiotic to be tested. Each tube containing the antibiotic dilution series was inoculated with 0,05 ml of a 16 hour old broth culture of the test strain. The titre was read after 24

Table III

Biochemical characteristics of the 52 E. coli O : 125 B : 15 strains isolated

	Adonite	Dulcitol	Sorbitol	Arabinose	Xylose	Rhamnose	Maltose	Salicine	Inositol	Lactose	Saccharose	Mannitol	Glucose	Galactose	Raffinose	Indol	Methyl-red	Voges-Proskauer	Ammonium citrate	H ₂ S	Ureum	KNO ₃	KCN	Gelatin	Movement	Polytrop Medium
Strains isolated	+	—	+	0	+	+	+	—	—	+	+	+	+	+	+	+	+	—	—	—	—	+	—	—	+	Typical
Type strains of																										
TAYLOR and CHARTER																										
A	0	+	0	0	0	0	+	—	—	+	+	+	+	0	0	+	+	—	—	—	—	0	0	—	—	0
B	0	+	0	0	0	0	+	—	—	+	+	+	+	0	0	+	+	—	—	—	—	0	0	—	—	0
C	0	+	0	0	0	0	+	+	—	+	+	+	+	0	0	+	+	—	—	—	—	0	0	—	—	0

Table IV

Results of quantitative antibiotic sensitivity tests of 10 strains of E. coli O : 125 B : 15

Name and inhibitory concentration of antibiotics			
Streptomycin	Chloramphenicol	Terramycin	Neomycin
12.5 to 6.25 µg	12.5 to 6.25 µg	2.5 to 12.5 µg	6.25 to 3.12 µg

hours' incubation at 37° C. The results of these tests are presented in *Table IV*.

The data of *Table IV* suggested the therapeutic importance of neomycin, as this antibiotic was found to be effective in a concentration of 3.12 µg/2 ml. The results obtained with streptomycin and chloramphenicol were also favourable, these antibiotics were effective in concentrations as low as 6.25 µg and 12.5 µg, respectively. Terramycin which in the qualitative test was effective, proved here weaker than the former drugs, as its effective concentration range was 25 to 12.5 µg.

Clinical observations. Of the 52 patients excreting *E. coli* O:125 B:15 thirty (60 per cent) had enteral symptoms, while the other 22 had not. The designation enteral symptoms meant a clinical entero-colitis and we differentiated 3, *i. e.* mild, intermediary and severe forms of the disease. The distribution according to severity is given in *Table V*.

Table V

Distribution of cases according to clinical severity

Enterocolitis								Symptom-free excretors		Total
mild		intermediary		severe		total				
No.	per cent	No.	per cent	No.	per cent	No.	per cent	No.	per cent	
18	34.6	9	17.3	3	5.8	30	57.7	22	42.3	52

As revealed by *Table V*, nearly two thirds (18) of our patients had mild enterocolitis. Those having an intermediary form of the disease amounted to 9, while only 3 cases could be regarded as severe.

The clinical picture of mild enterocolitis was characterized by a coated tongue, anorexia, 3 to 4 slightly mucous loose stools, 1 or 2 vomitings, sub-febrility. The symptoms lasted usually for 2 to 6 days. Only 3 of the patients were ill for more than 6 days. As to the age distribution, the youngest patient was 1 and a half month old, the oldest 3 and a half years. The average age was around 12 months.

Enterocolitis with intermediary symptoms was characterized by 8 to 6 watery, definitely mucous stools, frequent vomiting, a temperature of about 38° C and general weakness. The disease lasted usually for 5 to 15 days. A lag in weight development was regularly observed. Out of the 9 patients 5 were 1 to 6 months of age, while four 9 to 14 months of age.

Three of the patients exhibited severe enterocolitis, with 10 stools often combined with vomiting, a high temperature, sometimes hyperpyrexia and dullness. Body weight decreased sharply. The disease lasted usually for 8 to 12 days. No toxic manifestation was observed in any of our patients.

As a treatment, tetracycline intramuscularly or neomycin by mouth were prescribed. Fluid and electrolyte loss was replaced by infusions of plasma and Ringer-dextrose, later with repeated blood transfusions.

The number of excretors free of enteral symptoms was 22. Their age was between 2 and a half months and 4 years. They were isolated and subjected only to the treatment made necessary by their actual disease. Out of the 30 patients with enterocolitis only 12 had typical enteral symptoms at admission, while further 18 were admitted with upper respiratory infection. In the latter case the enteral symptoms manifested themselves either

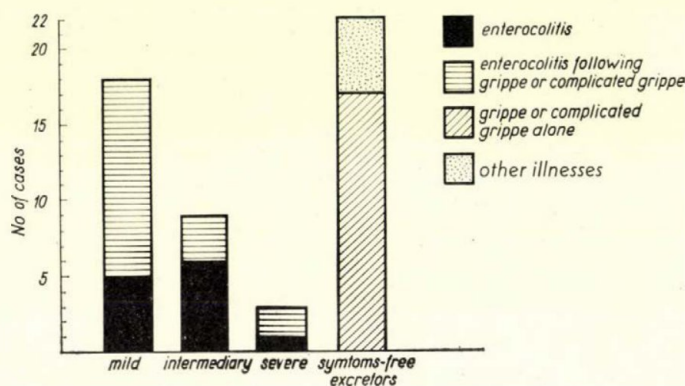


Fig. 1. Distribution of the 52 positive cases according to symptoms and severity of disease

simultaneously with the original disease or after recovery. Of the symptom-free excretors only 5 had no fever and catarrhal symptoms. These data are presented in Fig. 1.

Both the present studies and our earlier experience support the view that the disease produced by different *E. coli* strains manifested itself mostly after the organism's resistance had decreased in consequence of some extra-enteral illness (upper respiratory infection etc.). It appeared highly probable that apart from the massivity of infection, the virulence of the agent and the equilibrium of the enteral flora, the appearance and severity of clinical symptoms and the course of the disease was essentially influenced by the decreased resistance. The idea was further supported by the observation that severe, sometimes toxic acute gastroenteritis or enterocolitis occurred chiefly among marasmic, biologically weaker babies. Therefore it was thought important always to complete treatment with the administration of plasma, whole blood, gamma globulin and polyvitamin preparations, to help the organism to restore its original resistance. This supportive treatment was favourably influencing the disease in most cases.

Epidemiological data. As mentioned above, in the literature no other data than those of TAYLOR and CHARTER were found concerning the cumulated incidence of infection with *E. coli* O:125 B:15. As to the rarity of this strain let us quote the statistical data summarizing 6 years' (1950 to 1956) routine examinations at the Pasteur Institute of Lille [4]. They could isolate *E. coli* O:111 B:4 in 627 cases, while *E. coli* O:125 B:15 only in 3. Similarly at the Children's Hospital at Lille there were only two bacteriologically-verified cases of *E. coli* O:125 B:15 infection among 200 samples obtained within 3 years from children excreting different types of *E. coli* [4].

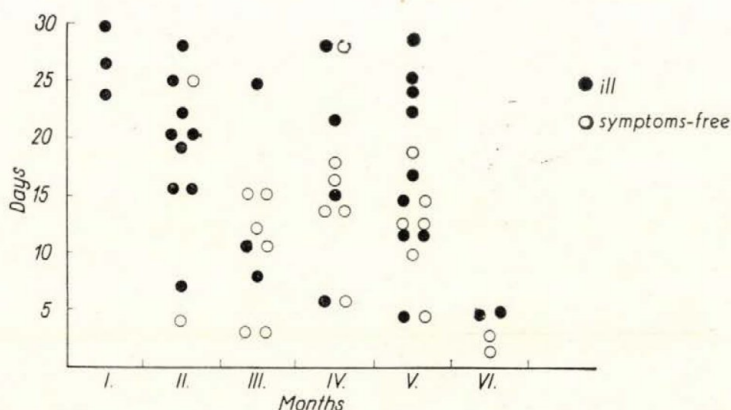


Fig. 2. Distribution of ill and symptom-free excretors according to date

The well-known fact that the overwhelming majority of *E. coli* epidemics were of nosocomial nature was true also for the present cases. It could not be a mere coincidence that soon after admission in January, 1959, of a baby infected with *E. coli* O:125 B:15 this extremely rare strain was so frequently isolated. Positive faecal samples were obtained from the contacts with simultaneously-developing symptoms of enterocolitis 3 to 8 days following admission while those remaining symptom-free produced positive faecal samples 2 to 4 days after admission. The incubation period of enteral disease caused by *E. coli* may greatly differ in length. BROWN observed incubation periods of between 2 to 20 days, OCKLITZ and SCHMIDT between 3 and 6, KREPLER and ZISCHKA between 3 and 33, DUPONT between 1 to 13 days. It has, however, been generally accepted that the minimum incubation was 48 hours, while the maximum seldom longer than 15 days. In our cases the incubation period for *E. coli* O:125 B:15 was 2 to 8 days and positivity lasted 2 to 10 days after the symptoms had subsided.

When surveying the nurses working in the babies' wards, we could isolate *E. coli* O:125 B:15 from the faecal sample of one nurse. Thus there was a possibility of this excretor having played a role in the spreading of the infection.

It is well-known that both the sporadic and epidemic incidence of *E. coli* in babies and children is strongly-influenced by age, the diet, premature birth and a number of different other factors.

The highest incidence of *E. coli* usually occurs during spring and autumn. The *E. coli* O:125 B:15 epidemic described by TAYLOR and CHARTER occurred during the autumn. The seasonal distribution of cases in the epidemic observed by us is presented in Fig. 2 in which the days of first isolation are plotted against the months.

According to the unanimous opinion of several authors enteral infections caused by *E. coli* are most frequent among infants of 1 to 8 months of age. The age distribution of the cases observed by us is presented in Table VI.

Table VI

Age distribution of patients and symptom-free excretors

	Age in months						Total
	1 to 3	3 to 6	6 to 9	9 to 12	12 to 18	18 to 48	
Patients	4	5	5	3	7	6	30
Symptom-free excretors	2	1	4	2	3	10	22

The increasing frequency of nosocomial *Coli dyspepsiae* epidemics have drawn attention to the possible role of environmental factors in spreading infection. In our Department we have observed all possible measures to avoid spreading of the epidemic. Faecal bacteriology is made of every admitted infant and child and in the case of a positive result the patient is transferred to a special ward where quite particular care is taken for hospital hygiene (hand disinfection, use of special dress, separate thermometers, washable toys and instruments, daily cleaning with disinfectants, etc.). The nursing staff is subjected systematically to bacteriological survey.

Discussion

The aetiological and epidemiological importance of *E. coli* O:125 B:15 in infants and children was first stressed by TAYLOR and CHARTER and the present study lends further support to their opinion, on the basis of clinical and epidemiological observations made in 52 cases of a hospital epidemic.

In our routine material of earlier years we had never found the strain in question. We first isolated it in January, 1959, from the faecal sample of a 6 months old infant suffering from enterocolitis. Following admission of this infant we repeatedly succeeded in isolating the strain during 5 months in con-

nection with a smaller nosocomial epidemic. During this period 52 positive infants and children were found. Among the positive infants and children some had more or less typical enterocolitis, while some of them were symptom-free. From the cases with typical enterocolitis (60 per cent) the organism could always be isolated in nearly pure culture even on repeated examination. Our attempts to isolate any other sort of pathogenic enteral organism from the same faecal samples invariably failed. The clinical and the epidemiological picture apparently induced by *E. coli* O:125 B:15 was similar to the pattern caused by the other known *E. coli* serotypes. The possible aetiological role of *E. coli* O:125 B:15 was further supported by the uniform antigenic structure, fermentation and antibiotic sensitivity of the strains which were isolated during the epidemic. The success of specific antibiotic therapy also supported the aetiological significance of the organism.

The comparatively high incidence (47 per cent) of symptom-free contact excretors appeared to be in contrast with the general experience in similar diseases. There is little doubt about the pathogenicity of *E. coli* O:111 B:4, although CATHIE [5] reported 30 per cent symptom-free excretors in epidemics caused by this organism.

Little is known about the pathogenicity of *E. coli* strains and the examinations of its virulence and toxicity — among others also those performed by us — did not yield the results expected. Thus there is a reason to suppose the existence of factors other than those inherent in the microorganism. One of these factors is probably the actual disposition of the host organism. Of the conditions unfavourably influencing the organism's resistance influenza, upper respiratory infections and some other extraenteral processes apparently deserve interest. The cumulated incidence of *E. coli* infections during the "catarrhal seasons" does not seem to be a more coincidence. This idea has been supported by our observation that most of the cases of enterocolitis had been encountered in patients suffering from upper respiratory infections. We thought it justified to conclude that *E. coli* O:125 B:15 usually producing secondary enteral infections may be regarded as a facultative pathogenic agent in infantile dyspepsia.

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Address of authors:

GYÖRGY UJVÁRY
Árpád út 126, Budapest IV, Hungary.

GÁBOR PÁLL
Ilka u. 57, Budapest XIV, Hungary.

THE ISOLATION AND SOME PROPERTIES OF THE COMPONENTS OF MYCOBACTERIUM TUBERCULOSIS

By

J. FÖLDES

Institute for Microbiology, University Medical School, Szeged

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Summary. New preparative methods have been developed for the isolation of some macromolecular components of *Mycobacterium tuberculosis*. Part of the lipids and lipoproteins were removed by acetone and phenol, and polysaccharide-type materials were extracted by a methanol-buffer mixture. The "bacterial residue" was solubilized by alkaline hydrolysis and the lipoproteins, lipoprotein-like complex compounds and the polysaccharides were fractionated. Some characteristic results obtained by the chemical analysis of the isolated and purified compounds are presented. DNA was found to be bound in increasing amounts to the successive fractions of ammoniumsulphate-precipitable lipoproteins. Depending on the proportion of alcohol used, a varying amount of nucleic acid resp. pentose was found to be associated with the polysaccharide fractions precipitated. Polysaccharides precipitated by two volumes of alcohol contained less pentose and more nucleic acid, while more pentose and less nucleic acid was found in polysaccharides precipitated by 5 volumes of alcohol.

Numerous literary data have been accumulated concerning isolation and characterization of the different components of the tubercle bacilli. A survey of these data was given by LONG [1] in an excellent review. It was clearly apparent from his paper that most of the authors had isolated and analysed only one or another single component of the bacterial cell, while some others had examined exclusively the tuberculin. ANDERSON [2] for example examined the components obtained by extraction with organic solvents, while SEIBERT [3] made extensive studies on the composition of tuberculin. STACEY [4] summarized in his review the studies on the polysaccharides of tubercle bacilli. Nevertheless up to now no sufficient interest has been devoted to the simultaneous isolation and examination of the macromolecular components of *Mycobacterium tuberculosis*.

Except the attempts made by HAWORTH *et al.* [5] no appropriately precise methods were available for the intracellular localization of the different substances isolated from tubercle bacilli. Recently the increasing amount of informations about bacterial anatomy [6] and the electron microscopic examinations on the structure of this organism [7, 8] have afforded new tools for the investigation into the intracellular localization of the different, chemically defined entities.

Keeping the above statements in mind we attempted simultaneously to isolate the macromolecular components of tubercle bacilli and to characterize the isolated materials as well as to localize them at least approximately in the

undamaged bacterial cell. As one should not disregard the specific substance of the MIDDLEBROOK—DUBOS-test [9], we had to choose methods essentially different from those used by others. The present paper will deal only with the details of our preparative methods and with some of the main characteristics of the materials isolated. Further details will be presented in forthcoming reports of this series.

Materials and methods

Bacterium strains. The H₃₇ Rv and the "Phylaxia" bovine strains were used throughout. The heat killed wet cell mass obtained from 8 weeks old Sauton cultures was supplied by the *Phylaxia Vaccine Institute, Budapest*, as a by-product of tuberculin manufacture.

Organic solvents. All organic solvents used for extraction were freshly distilled (acetone, phenol, methanol).

Aqueous solvents. The methanol saline solvent contained NaCl 0.55 per cent, Na₂HPO₄ 0.5 per cent and methanol 20 per cent in distilled water. The acetate buffer contained 2 per cent sodium acetate and 1 per cent glacial acetic acid in distilled water (pH 4.6).

Analytic methods. The following methods were used for characterizing the different materials isolated. The reducing materials expressed as glucose were determined by HAGEDORN and JENSEN's [10] method. The protein content was determined according to LOWRY [11], using an extinction curve obtained with a human serum of known nitrogen content. Quantitative determination of desoxyribonucleic acids (DNA) was by the diphenylamine method of DISCHE [12]. Pentoses were demonstrated by the orcinol test of MEYBAUM [13]. The lipid content of the isolated materials was estimated by FEIGL's "spot-test" [14]. 5 mg samples of the test material were hydrolysed with 0.1 N NaOH for 3 hours. The hydrolysate was extracted 3 times with ether, and the pooled extracts were evaporated. The residue was dissolved in .5 ml benzene. Determinations were carried out in 0.5 ml samples of this solution.

Results

Preparative methods. Lipids and lipoproteins were extracted from the moist bulk of tubercle bacilli by washing with acetone, extracting with phenol and treating again with acetone. Thus we did not follow the method of ANDERSON *et al.* [2], who first extracted very thoroughly with an equal mixture of ethanol and ether, then with chloroform, separated the ethanol ether-soluble lipids into acetone-soluble fats and acetone-insoluble phosphatides, and the material soluble in chloroform into methanol-soluble soft waxes and methanol-insoluble purified waxes. None of these fractions appeared to be homogeneous.

In our experiments 1 kg of moist bacterial cells were washed 3 times with 2 liters of acetone each, at room temperature under continuous stirring, for one hour every time. The pooled washing fluids (an aqueous acetone solution) were evaporated to 150 ml *in vacuo*. From the remaining aqueous solution a rusty brown material precipitated spontaneously. This was filtered through hardened filtre paper. The precipitate was re-dissolved in warm acetone and after cooling the small amount of butter-coloured insoluble material was

removed by centrifugation. The acetone was then evaporated. The remaining rusty brown pasty material amounting to 8.5 g was designated as "*Substance A*".

After acetone treatment the dry weight of the original bacterial mass was only 160 g.

The acetone-treated cell-mass was next extracted with 88 per cent phenol. Water-saturated phenol dissolves the lipoproteins from the surface and from the inside of the cell. WEIDEL and PRIMOSICH [15] supposed on the basis of their experiments with *E. coli* that phenolic extraction dissociates the two layers of the cell wall by dissolving the outer layer of lipoprotein character. Accepting this principle and following the description of MIDDLEBROOK and DUBOS [9], 50 g of the acetone-extracted cell-mass was treated 3 times with 1 liter of 88 per cent aqueous phenol solution each. A decreasing amount of yellowish-brown material was extracted by the successive phenol treatments. After centrifugation the treated cells were found to have sedimented, and a fat-like layer accumulated on the surface of the supernatant. This uppermost layer was collected and washed free of phenol by ethanol; it was designated "*Substance B*".

The fluid phase of the extract was dialysed against running tap-water. When the greatest amount of phenol was removed, a rusty brown precipitate was formed, with a tendency to aggregate, thus lending itself readily for separation. This was termed "*Protein I*".

The phenol-treated bacterial mass was repeatedly extracted with acetone (3 times 0.5 l each) partly to remove the phenol residues, partly to extract further lipids. The first washing was made with cold, the next two washings with warm acetone. After an overnight storage at 4° C a butter-like, white material precipitated from the pooled extracts. By filtration and drying a white powder was obtained, "*Substance C*".

After the extraction with phenol and acetone the dry weight of the bacterial residues was 37 g.

Extraction with acetone and phenol was followed by the extraction of the water-soluble components. Under the light microscope the cells at this phase were still appearing undamaged. The extraction was performed three times with each 500 ml of a methyl-alcohol-buffer mixture at 37° C for 8 hours under constant stirring. The extracts were pooled and fractionated by precipitation with alcohol. Adding two volumes of alcohol yielded a material which we termed *P I/a* and further 3 volumes of alcohol (a total of 5 volumes) another material designated *P I/b*.

The bacterial mass thoroughly extracted with the methanol buffer mixture was hydrolysed in 500 ml of 0.5 N NaOH. The hydrolysis was allowed to proceed at boiling temperature until the cells appeared to have totally dissolved. The hydrolysate was a brownish-yellow turbid colloidal solution. After cooling, the hydrolysate was centrifuged for 15 minutes at 3000 r. p. m.

resulting in the separation of 3 layers. On the surface a heavy soap-like crust termed "*Substance D*" was formed. The fluid phase was a brownish-yellow, turbid colloidal solution, while the sediment consisted of a small amount of sand-grey material, which was discarded. The protein- and polysaccharide-type substances were isolated from the fluid phase by further purification.

From the alkaline hydrolysate the acid precipitable proteins were isolated at pH 4 by the addition of an appropriate amount of acetic acid. The voluminous precipitate formed after two hours' standing was collected by centrifugation; it was designated "*Protein II*". The rusty brown-coloured, water-clear supernatants were pooled and neutralized.

The remaining non-acid-precipitable proteins were fractionated by ammonium sulphate. First 50 per cent saturation was made by adding 313 g ammonium sulphate per liter supernatant. After some hours of standing the precipitate was isolated by centrifugation: this was called "*Protein III*". The supernatant was then saturated to 75 per cent (by adding further 176 g ammonium sulphate per liter), when "*Protein IV*" was obtained. At 100 per cent saturation (further 189 g ammonium sulphate per liter) the protein precipitated was termed "*Protein V*".

The polysaccharides were fractionated with alcohol from the protein-free hydrolysate. Before alcoholic fractionation the bulk of ammonium sulphate was removed by dialysation against running tap-water for 48 hours. During dialysis the volume increased remarkably, and therefore the material had to be evaporated to 500 ml at 60° C *in vacuo*. The slightly lemon-coloured solution was buffered to pH 4.6 by the addition of 2 per cent sodium acetate and 1 per cent acetic acid. On the addition of 2 volumes of ethanol a polysaccharide fraction (*P II/a*) precipitated. On the addition of further 3 volumes of alcohol (total 5 volumes) the polysaccharide fraction designated *P II/b* was precipitated.

A schematic presentation of the above isolation procedures is given in *Fig. 1*.

Further purification of the water-soluble substances was made as follows. *P I/a* and *P I/b* materials were purified separately, though by the same method. The material was dissolved in 25 to 50 ml of acetate buffer. The insoluble residue was removed by centrifugation and the dissolved material was precipitated from the supernatant by the addition of ethanol. This procedure was repeated (usually 2 to 3 times) until a readily-soluble purified material had been obtained. After the last precipitation the material was washed with acetone. *P I/a* yielded a white, *P I/b* a yellowish white, dry powder. The total yield of *P I/a* plus *P I/b* substances was 1 to 2 per cent, depending on the actual batch.

P II/a and *P II/b* materials were purified in a way similar to that used for *P I/a* and *P I/b*. In this case, however, the removal of water-insoluble sub-

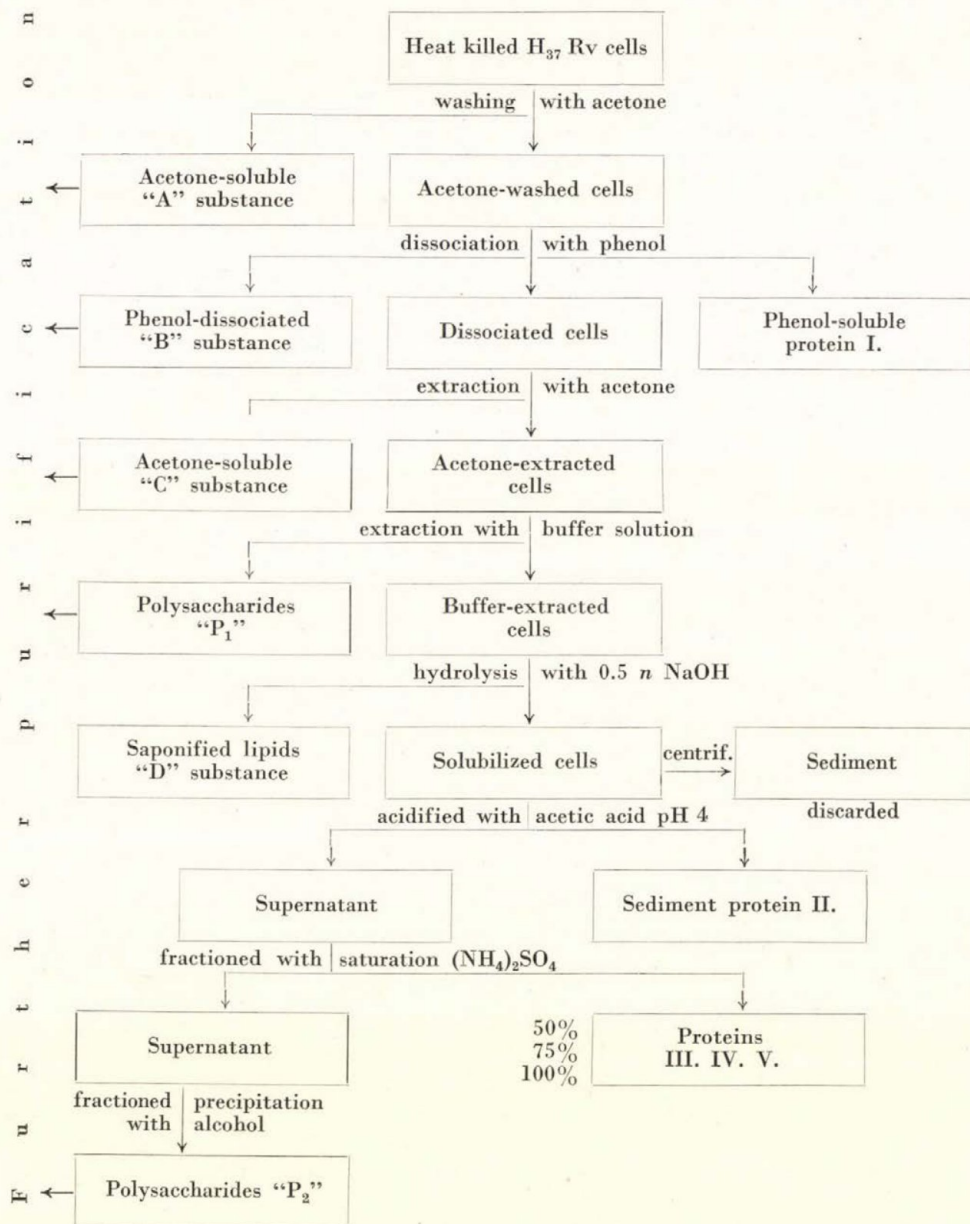


Fig. 1. Fractionation and isolation of the components of tubercle bacilli.
Schematic representation

stances was not directly followed by precipitation with alcohol. Instead, further purification was performed by adding some drops of a 10 per cent solution of $\text{Ba}(\text{OH})_2$ and the precipitate formed was removed by centrifugation. The excess amount of barium was precipitated by sulphuric acid. This was followed by dialysis against distilled water for 48 hours. Distilled water was changed at the 8th and 24th hours. The dialysed solution was either precipitated by the addition of alcohol or evaporated by a stream of warm air. The material obtained by either methods was then washed with acetone and dried in an exsiccator *in vacuo*. The final product from *P II/a* was a white, that from *P II/b* a yellowish-brown, powder. The total amount of *P II/a* and *P II/b* substances was 1.25 to 1.50 per cent of the starting material.

The acetic acid precipitated proteins (*Protein II*) were re-dissolved in 0.1 *N* Na_2CO_3 . The intensely opalescent solution was centrifuged to remove insoluble substances. The ivory-coloured sediment was discarded. The supernatant was acidified to pH 4 with acetic acid, and the precipitate formed was treated two additional times in the same way to achieve further purification. The substance obtained after the last purification was washed with a 2 per cent aqueous solution of acetic acid and with absolute alcohol. Finally it was dried *in vacuo*. Protein fractions obtained by fractionation with ammonium sulphate (*Protein III, IV, V*) were re-dissolved in distilled water neutralized by some drops of alkali. All protein fractions dissolved readily, yielding clear solutions golden yellow in colour. Further purification was achieved by dialysis. Dialysis was made against running tap-water for 24 hours, then against distilled water for 48 hours. The distilled water was changed at the 8th and 24th hours. The dissolved substances were precipitated by the addition of 5 volumes of ethanol. The precipitate was centrifuged, washed with absolute alcohol and dried in an exsiccator.

Characterization of the substances isolated. Data on the solubility of substances are presented in *Table I*.

Table I

Solubility of the substances extracted with organic solvents from M. tuberculosis H₃₇Rv

	Phenol	Chloroform	Ether	Acetone	Ethanol	Water
Substance "A"	s	s	s	s	s	i
Substance "B"	i	s	sl.s	i	i	i
Substance "C"	i	s	s	s	i	i

s = soluble

sl.s = slight soluble

i = insoluble

As revealed by the data in *Table I*, these substances can readily be differentiated on the basis of their solubility. Both substances A and C obtained by acetone extraction dissolved readily in acetone, ether and chloroform. It was, however, remarkable that in phenol and ethanol only substance A was dissolved, substance C appeared to be insoluble. Both substances were obtained by acetone extraction, though substance A was extracted before, while substance C after, treatment with phenol.

The rusty brown pasty substance A obtained by acetone extraction yielded on further fractionation 2 components. A colourless precipitate was formed on the addition of 2 volumes of water to the ethanol-dissolved substance A. The precipitate was collected on glass filtre or on hardened filtre paper (*Substance A_I*). The orange coloured substance that remained dissolved in the water-alcohol mixture could be shaken out with ether (*Substance A_{II}*). The ether-soluble fraction was evaporated until dry.

Data on the preliminary chemical analysis of the acetic acid and ammonium sulphate precipitated protein-like substances are presented in *Table II*.

Table II

Some characteristics of the protein components soluble in water from M. tuberculosis H₃₇Rv

	Protein %	Reducing materials %	DNA %	Lipids
Protein II	54.0	20.3	4.6	++++
III	62.0	22.8	7.0	++++
IV	42.5	26.3	30.5	++++
V	27.5	41.5	18.0	++++

DNA = desoxyribonucleic acid

The substances yielded by protein isolation methods were not homogeneous. Varying amounts of protein, carbohydrate-like reducing substances, DNA and lipids were found in the different fractions. It was remarkable that while the protein content of the successive fractions decreased, a parallel increase occurred in the protein-bound carbohydrate and DNA content. The protein content of "*Protein II*" precipitated by acid from the alkaline hydrolysate was 54 per cent. The amount of protein decreased successively in the further fractions obtained by precipitation with ammonium sulphate. In "*Protein V*" obtained by 100 per cent saturation with ammonium sulphate only 27.5 per cent protein, while 41.5 per cent carbohydrate and a high amount of DNA was found. Also the qualitative lipid reaction was strongly positive.

Data on the nature of the polysaccharide-type substances, isolated from the methyl-alcohol-buffer extracts, and of those precipitated by alcohol from the de-proteinized alkaline hydrolysate are presented in *Table III*.

Table III

Some characteristics of the isolated P (polysaccharide) materials from M. tuberculosis H₃₇Rv

Polysaccharides	Reducing materials %	Pentoses (RNA) %	DNA %	Lipids %
P I/a	45.2	15.5	35.0	++
P I/b	60.0	32.0	18.0	++
P II/a	73.0	19.6	4.0	++
P II/b	54.0	65.0	2.5	++

DNA = desoxyribonucleic acid

RNA = ribonucleic acid

The data summarized in *Table III* show that these extracts contained mostly carbohydrate-type substances. A varying amount of nucleic acids were found to be bound to the polysaccharides. When evaluating the analytical results, two interesting observations were made.

(i) The DNA content of the fraction obtained by precipitation with 2 volumes of ethanol was higher than that of the substance precipitated by the addition of further 3 volumes of ethanol. This was particularly remarkable in the case of substances *P I/a* and *P I/b* which in addition to the DNA contained some RNA. MEYBAUM's orcinol test as a positive colour reaction not being sufficiently specific for RNA as given also by different pentoses, to avoid non-specific reactions, spectrophotometric analysis in ultraviolet light was used for the quantitative determination of RNA in the presence of DNA in *P I* type substances [16].

(ii) Making use of the orcinol reagent, a very remarkable difference could be demonstrated in the amount of pentoses present in substances *P II/a* and *P II/b*. Considering that all *P II* materials had been isolated from alkaline hydrolysates, they did not contain RNA, *i. e.* the total amount of pentoses found had to be regarded as building stones of a polysaccharide (the sugar components of DNA reacts with orcinol to a negligible degree only). The pentose content of the polysaccharides precipitated with 2 volumes of ethanol was remarkably lower (19.6 per cent) than that obtained on the addition of further 3 volumes of ethanol (65.5 per cent). A more detailed analysis of these observations will be given in the next paper [16]. It seems to be worth of mention that in the polysaccharide-type extracts a moderate amount of lipids was demonstrated.

Discussion

Most authors when isolating the cellular components of tubercle bacilli aimed either at the preparation of an immunizing antigen or of a chemically well-defined substance of tuberculin activity. The chemical analysis of the cellular components yielded numerous, though widely variable results.

Up to now all attempts at preparing bacterium extracts possessing immunogenic activity have failed, although several authors succeeded in obtaining complex extracts with antigenic activity [17]. The tuberculo-proteins (PPD and PPD-S) prepared by SEIBERT are now generally accepted tuberculines.

Our knowledge about the substances contributing to the biological unit of tubercle bacilli is still unequivocal, partly owing to differences in the methods of preparation and partly because of the special metabolic characteristics of the tubercle bacilli. Both of these factors might be responsible for the observation that the different components occur in different amounts in different strains belonging to the same type. These variations are not only characteristic of the single strains but they might be observed within the same strain, depending on the age of the culture, the composition of the medium and certain other circumstances of cultivation [20, 21].

A schematic outline for the fractionated isolation of the component of tubercle bacilli was presented by RAFFEL [22] and LONG [1], mostly on the basis of the results of ANDERSON, SEIBERT, LEDERER and others. Studies on the composition of the bacterial cell followed two main directions, viz. (i) fractionation of the cell itself; and (ii) analytical studies making use of filtrates of synthetic fluid medium cultures. As the former method appeared to provide more reliable results, we decided to follow this way in our own experiments. We did not adhere strictly to any of the usual procedures and adapted the methods to our own aims. We intended simultaneously to isolate the macromolecular components of the bacterial cell and to analyse our preparations both chemically and serologically. Determining the intracellular localization of the substances thus isolated and characterized was also attempted.

The serologically active substances are usually isolated from lipid-solvent-treated tubercle bacilli. For the isolation of lipids the method recommended by ANDERSON *et al.* [2] is generally used. This method, however, does not only remove the fatty acids and rough waxes, but also the serologically active phosphatides. In our own experiments we used the phenol treatment [15] recently suggested for the study of the cell structure. Treatment with acetone was applied to dry the moist bacterial mass before phenol treatment, and also to remove the residual phenol after extraction. This treatment led naturally to the extraction of certain lipids, while the phosphatides insoluble in acetone remained intact in the cells.

Substance A obtained by acetone extraction could be separated into a colourless component A_I and a yellowish-brown one, A_{II} . We suppose that

A_{II} pigment is identical with phthiocol described by ANDERSON and NEWMAN [23]. The waxy *Substance C* obtained by extraction with warm acetone after treatment with phenol is different from *Substance A*, the former being insoluble in alcohol and phenol, while *Substance A* is readily dissolved by both solvents.

Phenol dissolves lipoproteins from the bacterial cell. After removing the phenol from the extract by dialysation, *Protein I* precipitates in the form of clumps rusty brown in colour. This denatured protein is dissolved only on sulphuric acid hydrolysis. The phenol extraction method was first applied by MIDDLEBROOK and DUBOS [9] for isolation of the specific substance of the haemagglutination test. It is highly probable from the experiments of WEIDEL and PRIMOSIGH [15] that the lipoprotein extracted is a component of the cell surface also in the case of tubercle bacilli, especially as after alkaline hydrolysis somatic lipoprotein could be isolated separately.

There are numerous methods available for further extraction of the "bacterial residue". Using aqueous-solvent mixtures one can extract from the lipid-free cell residues the polysaccharides, proteins, nucleic acids and firmly bound lipids as complexes of high molecular weight in a reasonably pure but never completely homogeneous form. Though the preparative methods differ remarkably in details, they might be grouped according to the actual pH used in the extraction procedure. Polysaccharides or proteins combined mostly with nucleic acids or lipids can be isolated by precipitation with an appropriate material (alcohol, acid, ammonium sulphate) from acid, neutral or alkaline extracts.

Extracts of bacterial residue obtained by extraction with dilute acid were studied by HEIDELBERGER *et al.* [24, 25], those extracted with alkaline solvents mainly by LAIDLAW and DUDLEY [26] and HAWORTH *et al.* [5]. For further details in this field the reviews of STACEY [4] and STACEY and KENT [27] should be consulted.

The somatic components of the tubercle bacilli can be isolated after an appropriate disruption of the cell. Of the methods used for this purpose, alkaline hydrolysis might be regarded as the crudest. Nevertheless, this method was used in our experiments as it seemed to be the most suitable for solubilizing all the structural elements and other components which might then be fractionated from the solution.

In our experiments the acetone- and phenol-treated bacterial residue was extracted with a methanol—saline mixture, with subsequent alkaline hydrolysis of the cells. The macromolecules (polysaccharides and proteins) isolated from both the aqueous extract and the alkaline hydrolysate appeared to be inhomogeneous. A great amount of carbohydrates, nucleic acids and lipids were bound to the protein-like extracts.

Remarkable differences were observed in the composition of polysaccharides isolated from the methylalcohol saline extracts and from the protein-free

alkaline hydrolysates. Thus from the extract designated *P I*, the polysaccharide precipitated on the addition of 2 volumes of alcohol contained twice as much DNA as that precipitated on the addition of further 3 volumes of alcohol. The pentose content of the same fractions varied in an opposite way. This significant difference in the pentose content obtained by precipitation with different amounts of alcohol was particularly characteristic of substances *P II*. The observation that in contrast to the 19.6 per cent pentose content of extract *P II/a*, *P II/b* contained 64 per cent pentose suggests the polydisperse character of the extracts as well as the influence of preparative methods on the character of the substances yielded.

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Address of the author:

JÓZSEF FÖLDES

Institute for Microbiology, University Medical School, Beloiannisz tér 10, Szeged, Hungary.

THE NATURE OF THE SPECIFIC POLYSACCHARIDES OF TUBERCLE BACILLI III

By

J. FÖLDES

Institute for Microbiology, University Medical School, Szeged

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Summary. The two types of polysaccharides isolated from tubercle bacilli were characterized by chemical, physico-chemical and serological methods. Attempts were made to establish their localization in the cell.

The polysaccharides isolated were serologically active. As determined by serological methods, these substances are sensitive to acid, while resistant to alkali. Ultracentrifugal analysis revealed the polydisperse character of both substances. Extract *P I* was found to be inhomogeneous and to contain considerable amounts of nucleic acids (DNA and RNA). Polysaccharide *P II* contains only a small amount of DNA. The *a* and *b* fractions of polysaccharides *P I* and *P II* differed in their pentose content, the difference was 2 to 3 fold. Both types of polysaccharides were found to contain amino-acids and amino-sugars, thus the cell wall origin of these substances appeared to be highly probable.

Our results together with the literary data suggest that polysaccharide *P I* may contribute to the outer layer of the cell wall, while polysaccharide *P II* to the internal one.

In an earlier study [1] we succeeded in isolating several different polysaccharides of *Mycobacterium tuberculosis* by fractionating certain extracts of heat killed, acetone- and phenol-treated cells. The polysaccharide extracted with a methyl alcohol — water mixture (pH 7.6) from the lipid free cells was composed of glucose and arabinose, while that obtained from alkali solubilized cells contained galactose and arabinose [2]. Both polysaccharides contained certain amounts of nucleic acid. Further examination of the above polysaccharides suggested their cell wall origin. An attempt was made to establish the possible intracellular localization of these polysaccharides on the basis of our analytical results and those obtained by others.

Materials and methods

Bacterium extracts. Polysaccharides were isolated as described previously [1, 3] from the bovine strain "Phylaxia" and *Mycobacterium tuberculosis* H₃₇Rv.

Immune sera. Rabbits were immunized through 3 weeks by 2 injections weekly with formalised H₃₇ Rv cell suspension. In the 4th week the animals were bled and the sera stored in the refrigerator until used.

Analytical methods. Determination of the reducing substances was made either by HAGEDORN and JENSEN's [3] method or by anthrone [5]. In both cases the amount of reducing substances was expressed as glucose. Estimation of pentoses was made with orcinol according to MEYBAUM [6], that of desoxyribose-nucleic acid (DNA) by the method of DISCHE [7].

Nitrogen and phosphorus were determined by the method of CLEGHORN and JENDRASSIK [8] and HARVEY [9], respectively. Amino-sugars were estimated by the modified method [11, 12] of ELSON and MORGAN [10]. The FEIGL's [13] spot-test was used for estimation of lipids. All quantitative determinations were performed in triplicate.

Chromatography. Material hydrolysed with sulphuric acid was used for the chromatography of carbohydrates. As a solvent butanol-pyridine-water (3 : 2 : 1.5) was used [14] and the chromatogram was developed with aniline-hydrogen-phthalate [15]. For amino-acid analysis material hydrolysed with hydrochloric acid was used. The chromatogram was run in a butanol-acetic-acid-water (40 : 10 : 10) mixture as described by MATTHIAS [16]. The amino-acid chromatograms were developed with ninhydrin, amino-sugar chromatograms with silver nitrate [17].

For spectrophotometry* the Beckman DU photoelectric spectrophotometer was used. Ultracentrifugal studies* were made in a *PHYWE*-apparatus.

Serological methods. For haemagglutination tests sheep-erythrocytes stored in ALSEWER [18] solution were used. The MIDDLEBROOK—DUBOS test [19] was performed in TAKÁTSY's Microtit-apparatus [20] as described earlier [2]. In the present experiments 0.025 ml volumes were used.

For the precipitation tests the antigen dilutions were prepared in 0.4 ml volume, adding the same amount of serum.

The agar diffusion method was performed as described by SEIBERT [21].

The acid and alkali sensitivity of the polysaccharides was examined as follows. Samples of a 100 mg per 100 ml solution of the polysaccharide examined were hydrolysed in 0.1 *N* NaOH or HCl in a boiling water bath. The serological activity of the above hydrolysates was examined by sensitization and agglutination inhibition tests in neutralized samples taken at different points of time.

Results

The polysaccharides isolated differed in serological activity. The differences between *P I* and *P II* substances could best be demonstrated by the Middlebrook-Dubos test. *P I* type extracts sensitized the erythrocytes, while *P II* polysaccharides did not [2]. Nevertheless both polysaccharides were able to combine with antibody, and to inhibit agglutination of the sensitized red blood cells.

In Table I a quantitative presentation of the above relations is given.

Table I

Serological activity of polysaccharides isolated from M. tuberculosis as estimated by haemagglutination inhibition

Antigen	Dilution in thousands							
	2	4	8	16	32	64	128	256
P I/a Human	—	—	—	—	—	+	+++	++++
P I/b Human	—	—	—	—	—	+	++++	++++
P II/a Human	—	—	—	+	+++	++++	++++	++++
P II/b Human	—	—	—	—	—	+++	++++	++++
P I/a Bovine	—	—	—	—	+	+++	++++	++++
P I/b Bovine	—	—	—	—	++	++++	++++	++++
P ₂ Bovine	—	—	—	—	—	—	+	+++

By the use of a serum of 16 haemagglutinating units (HU). P₂ polysaccharide was precipitated (without fractionation) with 5 volumes of alcohol.

* Measurements were performed by the University Central Laboratory. The author is grateful for the valuable help.

Figures in *Table I* show that both polysaccharides have a serological activity of approximately the same order of magnitude, *i. e.* 32 000 to 128 000 fold dilutions of the "antigen" inhibited the haemagglutinating effect of a serum dilution containing 16 haemagglutinating units (HU).

A similar conclusion was drawn from the results of the precipitation test.

Table II

Serological activity of polysaccharides isolated from M. tuberculosis as estimated by precipitation test

Antigen	mg/ml			
	1	0.1	0.01	0.001
P I/a Human	++++	++	—	—
P II/a Human	++	+++	+++	++
P II/b Human	++	+++	+++	++
P I/a Bovine	+++	+	—	—
P ₂ Bovine	++	+++	++++	++

Data presented in *Table II* show that rabbit immune sera were precipitated by the polysaccharides. *P II* substances were active even at dilutions of 1 to 1 000 000 (0.001 mg per ml), while *P I* type polysaccharides had remarkably less activity.

Data concerning the alkali- and acid-sensitivity of our polysaccharide preparations are presented in *Figs. 1* and *2*.

By treatment with 0.1 *N* alkali or acid at 100° C the polysaccharides were found to suffer characteristic damages. Partial acid hydrolysis destroyed the serological activity of both polysaccharides within 7 to 15 minutes under the experimental conditions used. Alkaline hydrolysis on the other hand de-

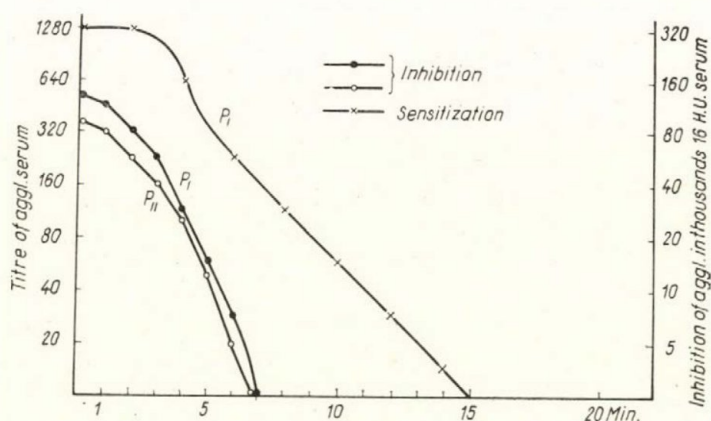


Fig. 1. Acid sensitivity of polysaccharides isolated from *M. tuberculosis* (0.1 normal hydrochloric acid at 100° C)

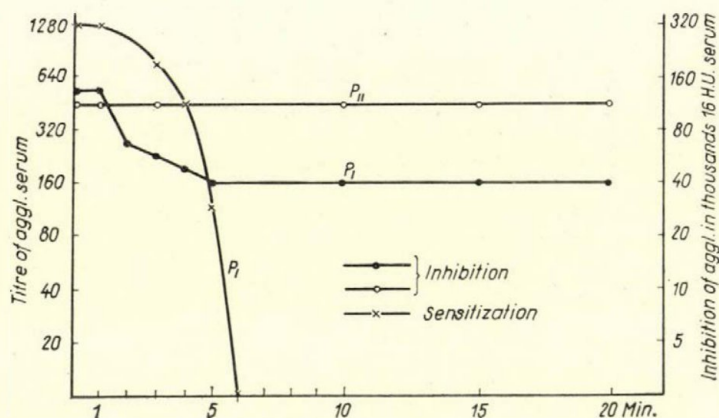


Fig. 2. Alkaline sensitivity of polysaccharides isolated from *M. tuberculosis* (0.1 normal sodium hydroxide at 100° C)

stroyed only the sensitizing activity of substance *P I* within 6 minutes. The agglutination inhibiting activity of substance *P II* was unchanged and after a moderate decrease the same was observed for substance *P I* as well.

In some earlier fractionation experiments performed with lanthanum salts [2] the *P I* type substances appeared chemically inhomogeneous. After the precipitation of nucleic acid (NA) half to one third of the initial polysaccharide content was found in the supernatant. When tested by serological methods the polysaccharides were entirely homogeneous. This latter observation was supported also by our recent agar diffusion experiments. Using the double diffusion method described above, one boundary was demonstrated for each of the two polysaccharides. Precipitation was optimal at concentrations of 1 mg per ml with *P I* and 0.01 mg per ml of *P II*.

The PHILPOT curve obtained by ultracentrifugation analysis revealed the polydisperse nature of the substances. Our pertaining observations might be summarized as follows. At 15 000 r. p. m. 2 components could be observed in *P I/a* extracts. At 50 000 r. p. m. the first component was entirely sedimented, the second still under sedimentation and the flotation of a third component could also be demonstrated. In *P I/b* extract only one sedimenting component was observed at 15 000 r. p. m. while at 50 000 r. p. m. the amount of floating substances was greater than in *P I/a*. The ultracentrifugal analysis of *P II/a* polysaccharide revealed only one single component at 50 000 r. p. m. In polysaccharide *P II/b* two components were observed at 50 000 r. p. m.; the principal component was of a lower molecular weight than the other component. Ultracentrifugal analysis of substance *P₂* yielded a sum of the results obtained with substances *P II/a* and *P II/b*; at 50 000 r. p. m. three components were observed, two of higher molecular weight in small amounts and one of lower molecular weight in a greater amount.

Table III

Chemical analysis of the polysaccharides isolated from M. tuberculosis

	Reducing substances		Pento- ses	Hexos- amine	N	P	Nucleic acids	
	Hage- dorn- Jensen	An- throne					DNA	UV. 260 $m\mu$
	Per cent							
<i>P I/a</i> Human	70.0	62.5	10.5	4.4	3.4	1.5	12.5	17.5
<i>P I/b</i> Human	48.0	47.5	28.0	4.8	2.1	0.8	7.1	9.7
<i>P II/a</i> Human	73.0	70.0	19.6	3.3	1.1	0.67	3.0	3.0
<i>P II/b</i> Human	54.0	55.0	65.0	3.75	1.25	0.37	1.5	1.7
<i>P I/a</i> Bovine	45.0	35.0	15.0	2.8	7.4	4.0	35.0	45.0
<i>P I/b</i> Bovine	60.0	45.0	32.0	3.75	3.2	1.83	17.0	19.5
<i>P</i> ₂ Bovine	65.0	42.0	41.0	4.0	2.9	0.4	5.0	4.4

Results of the chemical analysis of the substances are given in *Table III*. From the results the following conclusions were drawn.

(i) The extracts were inhomogeneous. As revealed by the amount of reducing substance the extracts contained mostly polysaccharides containing more or less nucleic acids. The nucleic acid content of *P I* substances was comparatively high.

(ii) Values obtained by the Hagedorn—Jensen method and with the anthrone reagent were different. This difference was explained by the nature of the anthrone reagent [22] and at the same time provided information on the nature of the building stones of the polysaccharide [23].

(iii) The differences in the pentose content as estimated with the orcinol reagent in the substances precipitated with fractionation *P I/a*, *P I/b* and *P II/a*, *P II/b* were remarkable. The pentose content of the polysaccharides precipitated by 2 volumes of alcohol was only one half or one third of that contained by the fractions precipitating on the addition of further 3 volumes of alcohol.

(iv) All the polysaccharides contained nucleic acids. The spectrophotometric analysis revealed an adsorption maximum at 264 m μ wave length in both polysaccharides. The highest nucleic acid content was demonstrated in the *P I* extracts. The difference in DNA content as estimated by the Dische test and the total nucleic acid content as measured by the adsorption at 264 m μ suggested the amount of RNA being rather low. Extracts *P II* obtained with alkaline hydrolysis did not contain any RNA and the amount of DNA was also very low.

(v) The phosphorus content of the extracts varied parallel to that of nucleic acids. The variations in nitrogen content were also parallel to the

former, but at a higher level. This difference might have been due to the presence of hexosamines and amino acids demonstrated also by chromatography. The positivity of the qualitative carboxyl acid ester test also suggested the presence of lipids.

Chemical analysis, however, did not supply any data on the structure of the polysaccharides, nor on the precise nature of their single components. Though we possess some data concerning the composition of extracts P_1 and P_2 [2], it still seems necessary to perform more detailed studies on *a* and *b* polysaccharide fractions exhibiting remarkable differences in their pentose con-

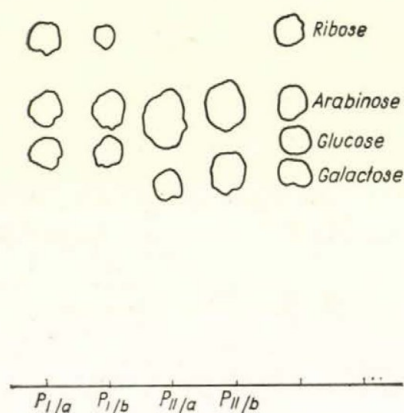


Fig. 3. Chromatogram of a sulphuric acid hydrolysate of the polysaccharides isolated from *M. tuberculosis*

Hydrolysis in 4 normal sulphuric acid for 6 hours. Chromatography was performed in butanol-pyridine-water (3 : 2 : 1.5) developed with aniline hydrogen-phthalate

tent. The chromatograms presented in Fig. 3, added interesting data to those mentioned above.

As revealed by Fig. 3, $P I/a$ and $P I/b$ extracts contain essentially the same carbohydrate components. The spots observed on the chromatogram were identified with standard sugars as glucose, arabinose and ribose, the presence of ribose being a sign for the eventual presence of nucleic acids. The composition of $P II/a$ and $P II/b$ substances was also similar, both contained galactose and arabinose. Thus the differences between fractions *a* and *b* were probably more of quantitative than of qualitative nature.

The nitrogen contents of the extracts were higher than calculated from the amount of nucleic acid present. This observation might have been due both to the presence of a hexosamine component and to that of certain other nitrogen-containing substances. Chromatograms of the hydrochloric acid hydrolysed materials were developed partly by ninhydrin, partly by silver nitrate.

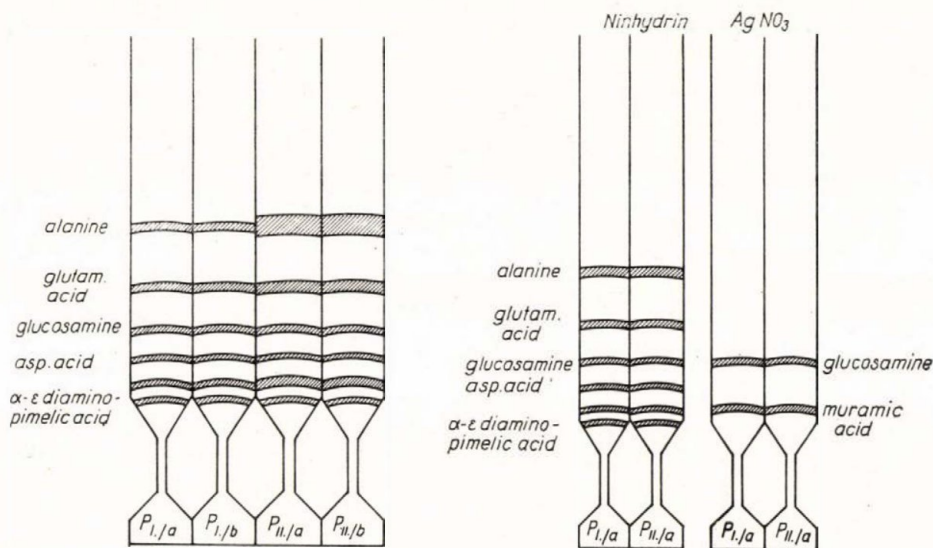


Fig. 4. Chromatogram of the hydrochloric acid hydrolysate of the polysaccharides isolated from *M. tuberculosis*

Hydrolysis in 4 normal hydrochloric acid for 10 hours. Chromatography was performed in butanol-acetic acid-water (4 : 1 : 1), developed with ninhydrin or AgNO_3

The ninhydrin developed chromatogram revealed the presence of certain amino acids, suggesting the presence of a peptide residue in the polysaccharide extract. The peptide residue was found to be composed of alanine, glutamic acid, aspartic acid, α, ϵ -diamino pimelic acid, glucosamine and probably muramic acid (3—0— α -carboxy-ethylhexosamine) [24]. The amino acids were identified by control materials run in parallel.

Evaluating the analytical results obtained, the polysaccharides isolated appeared to have originated from the cell wall. This supposition was further supported by the demonstration of α, ϵ -diamino-pimelic acid and the possible presence of muramic acid; both substances are known to be specific for the cell wall material. Our findings were in harmony with those of CUMMINS and HARRIS [9, 5]. These authors isolated by mechanical methods the cell wall of the tubercle bacilli and of many other bacteria, and their analytical data essentially agreed with those of the *P II* extracts prepared in our laboratory. The presence of the amino acids and amino sugars demonstrated also in *P I* extracts suggested their cell wall origin, though the polysaccharide was composed of glucose and arabinose. A polysaccharide of similar composition was isolated by ANDERSON *et al.* [26] from the acetone insoluble phosphatide fraction. Later HAWORTH [27] succeeded in isolating two different, hexosamine-containing compounds which he termed lipid-bound and somatic polysaccharides. The outermost coat of the bacterial cell, the so-called cord factor, is a petrol-ether soluble ester of mycolic acid and trehalose [43]. Thus glucose

containing compounds may apparently contribute to the structure of the outermost layers of the bacterial cell.

The two polysaccharides of cell-wall origin were isolated by different methods. *P I* type polysaccharides were isolated by extraction with a methyl-alcohol-saline mixture of pH 7.6. After this procedure the cells appeared to be structurally undamaged when examined under the light microscope. It was supposed therefore that by this method only the outer loose layer of the cell wall had been dissolved and analysed. Another type of polysaccharide of cell-wall origin was isolated by fractionation of the alkaline hydrolysate of the solubilized cells. The schematic localization of substances *P I* and *P II*, as

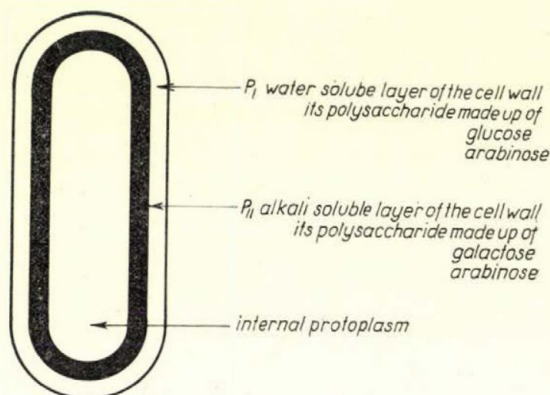


Fig. 5. Schematic representation of the structure of tubercle bacilli

revealed by the methods of preparation, the nature of the isolated substances, and the literary data available are presented in Fig. 5.

According to Fig. 5, extract *P I* contained materials from the outer coat of the bacterium, while polysaccharide *P II* was a component of the internal rigid frame of the cell wall. Our studies on the complex materials contributing to the composition of the protoplasm will be presented in a separate paper.

Discussion

According to their origin, the polysaccharides isolated from tubercle bacilli may be divided in 3 groups [29]: (i) polysaccharides from the somatic elements of the cell; (ii) polysaccharides isolated from the lipid fractions; and (iii) the polysaccharides of "tuberculin". According to their composition, all these polysaccharides appear to belong to two types, (a) polysaccharides composed of arabinose and mannose; (b) polysaccharides containing galactose in addition to these, two sugars. Some authors reported to have found beside the above monosaccharides small amounts of amino-sugars [30] and rhamnose. Galactose was found only in polysaccharides obtained by alkaline extraction.

CUMMINS and HARRIS [25] demonstrated the presence of a polysaccharide composed of arabinose and galactose in the isolated cell wall of the tubercle bacillus. The polysaccharides present in fraction *P II* contained the same sugar components. Glutamic acid, alanine, aspartic acid, α,ϵ -diaminopymelic acid, glucosamine and muramic acid, the substances characteristic of the cell wall of Grampositive bacteria, were also found in fraction *P II*. These data seemed to justify our supposition concerning cell-wall origin of *P II* polysaccharide. The similar amino acid and hexosamine content of extract *P I* made the cell wall origin of this substance also highly probable. *P I* polysaccharide, however, seemed to be different from the generally-known mannose and arabinose containing polysaccharides, being composed of arabinose and glucose. The presence of glucose in both extract *P I* and the cord factor pointed to its eventual role as a building stone of the structural elements of the cell surface. Let us mention that the R_f value of glucose and mannose is nearly similar, and that the epimer character of these two sugars should always be kept in mind whenever they are to be identified in parallel.

The differences in the composition of *P I* and *P II* polysaccharides suggest the cell wall in its broader sense to consist of more layers. This view is based not only on our own findings but also on some morphological and chemical analyses made by other authors. KNAYSIS [31] demonstrated by electron microscopy a double marginal layer on the surface of tubercle bacilli. BASSERMANN [32] made similar observations and described a third additional outermost layer, demonstrable under special conditions only. Electron microscopy of organisms other than tubercle bacilli also suggested the structured nature of the cell wall. Two layers of the cell wall of *B. megatherium* were demonstrated by electron microscopy by CHAPMANN [33]. KELLENBERGER and RYTER [34] made similar observations on *E. coli*. The double layered structure of the isolated cell wall of *Beggiota* bacilli (spirillum species) was demonstrated on electron micrographs by HOUWINK [35].

Chemical analysis yielded further proofs of the complex structure of the cell wall. Two different types of polysaccharide were obtained from purified yeast cell wall preparations by NICKERSON *et al.* [36, 37]. Of the two types of polysaccharide isolated those of the glucane type appeared to form the outer layer of the cell wall, while those of the mannane type the inner one.

Nucleic acids were present in both of our polysaccharide preparations, especially in extracts *P I*. It seems necessary to decide whether these nucleic acids are bound to the polysaccharides isolated or should be regarded as contaminants. Our earlier fractionation experiments together with serological and chemical examinations [2] suggested these nucleic acids to be firmly associated with the polysaccharides.

As to the role and presence of nucleic acids and nucleotides in the cell wall, there are several data available. In contrast to SALTON and HORNE [38],

BARCULIS and JONES [39] were able to demonstrate an absorption maximum at 260 $m\mu$ in acid hydrolysates of purified *Streptococcus faecalis* cell walls. A new type of ribonucleotide, teichoic acid was demonstrated in the cell wall of a number of bacteria by BADDILEY [40]. This new ribonucleotide was found in high amounts (40 to 60 per cent) in certain cell wall preparations. Further suggestive results and studies on the role of nucleotides in the synthesis of the cell wall were published by PARK and JOHNSON [41] and STROMINGER [42].

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Address of the author:

JÓZSEF FÖLDES

Institute for Microbiology, University Medical School, Beloiannisz tér 10, Szeged, Hungary.

CHEMICAL AND SEROLOGICAL INVESTIGATIONS ON THE PROTEINS OF MYCOBACTERIUM TUBERCULOSIS

By

J. FÖLDES

Institute for Microbiology, University Medical School, Szeged

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Summary. In previous papers we have reported about the isolation of macromolecular components of tubercle bacilli, and the studies on the polysaccharides isolated. The present report deals with the protein-like substances found.

Protein fractions II to V are complex macromolecules, containing polysaccharides and nucleic acids bound to protein. They are present in gradually increasing amounts in the successive fractions. The extracts seem to contain also some lipids.

As revealed by chromatographic analysis the protein contains at least 12 different amino acids. The polysaccharide component contains arabinose, glucose and galactose. The nucleic acid present appeared to be exclusively DNA.

The extracts were serologically active; they precipitated the homologous rabbit immune sera and neutralized the antibody in the MIDDLEBROOK—DUBOS haemagglutination inhibition test. They did not sensitize normal red blood cells, while they adsorbed to those pretreated with tannic acid. The serological activity appeared to be directly proportional to the polysaccharide content of the extracts.

By the agar diffusion method the extracts appeared to be homogeneous, as judged from the presence of a single precipitation boundary. In ultracentrifugation experiments the macromolecules exhibited polydispersity.

The substances of protein character isolated seem to contribute to the composition of the cellular protoplasm.

Up to now the proteins of tubercle bacilli have been studied mostly from an immunological point of view. In this type of experiments the protein components of tuberculin were mostly examined, and less attention has been paid to proteins isolated directly from intact normal cells [1, 2]. As a result of similar experiments several protein substances of great practical importance were prepared by different authors, using different methods; *e. g.* PPD [3] and PPD-S [4], the A, B and C proteins of SEIBERT [5] and the substances isolated by STACEY [6] and HECKLEY and WATSON [7]. The composition and nature of these proteins appeared greatly to depend on the method of preparation, *i. e.* on whether the protein had been isolated from autoclaved or living cells, by rough or mild methods.

In the course of our attempts to fractionate the macromolecular components of tubercle bacilli, materials of protein nature were also isolated [8]. It has been pointed out in the preceding papers that our aim was to isolate the macromolecular components in a way allowing at the same time to establish their probable intracellular localization. In the present paper we shall report on our results obtained by chemical and serological analysis of the crude and

purified "protein" preparations obtained in our experiments. On the basis of these data a tentative localization of the materials isolated has also been attempted.

Materials and methods

Isolation of the proteins was made as described earlier [8].

Immune serum. Rabbits were immunized as described previously [9].

Sheep erythrocytes stored in Alsewer solution [10] were used.

Serological examinations. A micro-modification of the MIDDLEBROOK—DUBOS test was performed, as described earlier [11]. Precipitation tests were made partly by the classical method in 0.8 ml volume and partly by the agar diffusion method described by SEIBERT [12].

Analytical methods. Protein determination was made with FOLIN's phenol reagent as described by LOWRY [13]. Nitrogen was determined by NESSLER's reagent as suggested by CLEGHORN and JENDRASSIK [14]. Phosphorus was determined by the method of HARVEY [15]. The amount of protein-bound DNA was estimated with the diphenylamine reagent according to DISCHE [16]. For estimation of the lipid content FEIGL's spot-test [17] was used, as described earlier. The amount of reducing substances was estimated by the HAGEDORN—JENSEN method [18].

Paper chromatography was performed after hydrolysis with sulphuric acid or hydrochloric acid. After hydrolysis superfluous sulphuric acid was removed by precipitation with BaCO_3 , hydrochloric acid by evaporation *in vacuo* over NaOH. The hydrolysates were run on Whatman No. 1 paper in butanol-pyridine-water (3 : 2 : 1.5) mixture [19] or in butanol-acetic-acid-water (4 : 1 : 1) mixture [20]. The chromatograms were developed with ninhydrin and aniline-hydrogen-phthalate, respectively. For further details our previous papers should be consulted.

*Spectrophotometric examinations** were performed with a Beckman DU photoelectric spectrophotometer, using distilled water as a solvent.

*Ultracentrifugation studies.** The determination of molecular dispersity of the protein solutions obtained was made by the use of a Phywe (Göttingen) ultracentrifuge and the results were evaluated on the basis of the Philpot curve.

Results

Solutions of proteins precipitated by fractionation with ammonium sulphate were dialysed and precipitated with alcohol. A preliminary analysis of the materials was made before alcohol precipitation. *Figs. 1* and *2* show the optical density of our preparations of different grades of purity. The measurements were carried out in ultraviolet light at a wavelength range between 3200 and 2200 Å.

As revealed by the curves the dialysed crude extracts contained substances having an absorption maximum both at 2800 Å and 2600 Å. In the protein preparations *II* and *III* proteins with an absorption maximum at 2800 Å formed the majority, while in *Proteins IV* and *V* a marked absorption maximum occurred at 2640 Å. This was considered indicative of the presence of great amounts of nucleic acid. In *Fig. 2* curves obtained by spectrophotometry of alcohol-precipitated substances are presented. As compared to *Fig. 1*,

* The measurements were performed by the University Central Laboratory. The author is grateful for the valuable help.

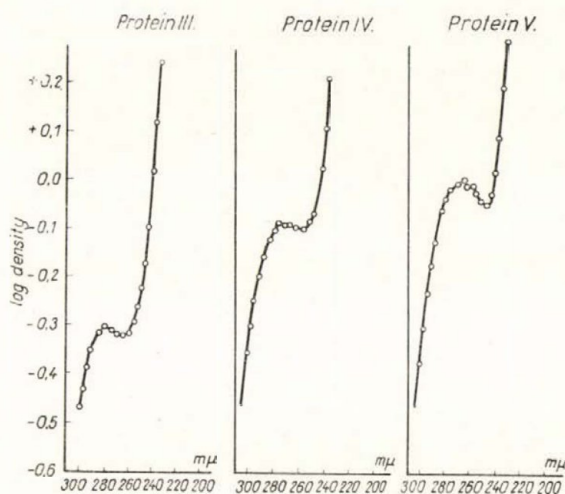


Fig. 1. U. V. absorption of proteins obtained by fractionation with ammonium sulphate

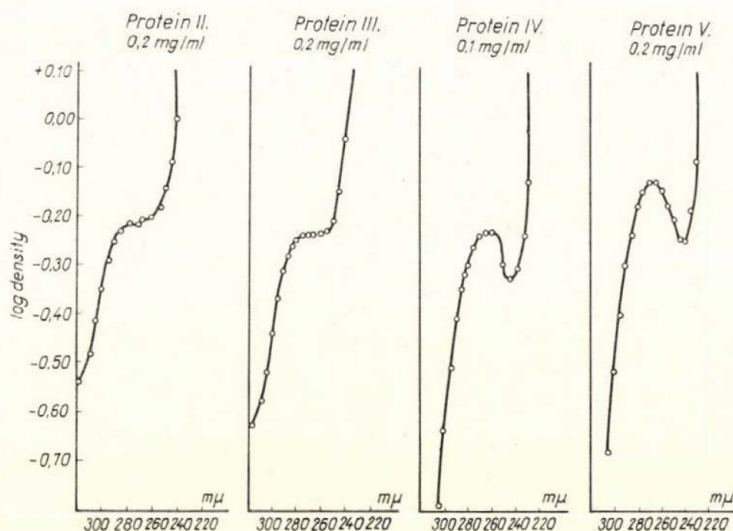


Fig. 2. U. V. absorption of proteins after purification by alcohol precipitation

no remarkable change in the shape of the curves could be observed, though the absorption maximum at 2640 Å was distinct.

Chemical analysis supplied further support and some additional details to the data revealed by ultraviolet absorption studies. The results are summarized in *Table I*.

Table I

Chemical components of proteins isolated by the fractionation of M. tuberculosis H₃₇Rv

	Reducing substances per cent	Protein	DNA	N	P	Lipid test
		Per cent				
Protein II	20.3	54.0	4.6	12.5	0.35	++++
III	22.8	62.0	7.0	14.5	0.46	++++
IV	26.3	42.5	30.5	14.5	2.01	++++
V	41.5	27.5	18.0	9.0	1.5	++++

The data in *Table I* showed that neither the acid-precipitated protein fractions nor the ammonium-sulphate-precipitated protein fractions were homogeneous. The materials isolated appeared to be composed of varying amounts of protein, polysaccharides and nucleic acids; *e.g.* fraction *Protein II* contained 54 per cent of protein, 20.3 per cent reducing substances and a small amount (4.6 per cent) of DNA. In the further fractions the amount of reducing substances increased successively, while that of the proteins decreased in parallel. The DNA content of *Proteins IV* and *V* was remarkably high. The determined amount of N and P was in a good approximation to the calculated N and P content of the proteins and DNA present. The presence of lipids in every extract appeared to be interesting.

The protein-polysaccharide ratio in extracts purified by dialysis only, exhibited variations similar to those revealed by the analytical data; the relative predominance of the polysaccharide components, *i. e.* the decrease of the numerical value of the ratio was accompanied by a parallel increase of the precipitin titre [21].

Precipitin tests carried out with the isolated substances yielded further information as to their composition. The results are presented in *Table II*.

Table II

Precipitating activity of proteins isolated from M. tuberculosis H₃₇ Rv

Antigen	mg/ml			
	1	0.1	0.01	0.001
Protein II	+++	—	—	—
III	+++	++	+	—
IV	++++	+++	++	+
V	++++	+++	+++	+

The data of *Table II* suggest a close correlation between polysaccharide content and precipitating activity of the extracts. Results of MIDDLEBROOK—DUBOS haemagglutination-inhibition tests yielded further support to this observation, as demonstrated in *Table III*.

Table III

*Inhibition of haemagglutination by proteins isolated from M. tuberculosis H₃₇ Rv**

	Dilution of antigens in thousands							
	2	4	8	16	32	64	128	256
Protein II	—	—	++	+++	+++	++++	++++	++++
III	—	—	++	+++	++++	++++	++++	++++
IV	—	—	—	+	++	+++	++++	++++
V	—	—	—	—	—	++	+++	++++

* Red blood cells sensitized with P I/a

As revealed by the data presented in *Table II* and *III*, the serological activity of the single extracts increased parallel to the amount of polysaccharide bound to protein antigen. The serological tests were carried out using an appropriate rabbit immune serum. In the complex extracts isolated the only free, serologically reactive groups were those of the polysaccharide, while those of the protein appeared to be masked. This supposition might furnish an explanation of the results of quantitative precipitation and red blood cell sensitization tests. Experiments by the latter method are presented below.

We supposed that our protein-like substances might sensitize red blood cells if the haemagglutination test was made according to BOYDEN's modification [22]. Tannic-acid treated cells are known to adsorb proteins and thus to become agglutinable by an appropriate immune serum. The protein extracts obtained in our experiments were also found to be adsorbed on tannic acid treated cells [33]. On the other hand, our substances inhibited agglutination of red blood cells sensitized with *P I* polysaccharide. This suggested that (i) for the serological activity of the complex molecule the polysaccharide bound to the protein was responsible; (ii) that the specificity of red blood cells coated with *P I* extract or old tuberculin was inferred by the polysaccharide; (iii) that in the haemagglutination inhibition test the extracts *Protein II* to *V* exerted their inhibitory effect by neutralizing the antibody specific for the sensitizing polysaccharide.

The serological homogeneity of our substances was tested by the agar diffusion method. For these tests sera of rabbits immunized with formalinized tubercle bacilli were used. This serum contained anti-polysaccharide antibody as demonstrated in our earlier experiments [9]. No data on the eventual pres-

ence of other specific antibodies in the serum were available. Results of the agar diffusion experiments are presented in *Table IV*.

Table IV
Localization of the precipitation boundaries at different antigen dilutions

mm from serum meniscus	Protein II mg/ml				Protein III mg/ml			
	1	0.1	0.01	0.001	1	0.1	0.01	0.001
1	++				++			
2								
3		++				+++		
4								
5			++++				++++	
6				++				
	Protein IV				Protein V			
1	++				++			
2								
3		++				+++		
4			++++				++++	
5				++				
6								

Clear-cut precipitation was obtained at a concentration as low as 0.01 mg per ml. All the complex extracts gave only one single precipitation boundary independently of their concentration actually tested. Thus they appeared to be serologically homogeneous.

In contrast to the serological homogeneity, ultracentrifugal analysis revealed a molecular polydispersity of our materials. This fact naturally does not exclude the possibility of their electrophoretic homogeneity and *vice versa* [23].

Ultracentrifugal analysis of our protein extracts revealed the following characteristics: at 20 000 r. p. m. from *Protein II* 2 components sedimented and further 4 components at 50 000 r. p. m. The main component had the lowest molecular weight. In *Protein III* rapid sedimentation of 1 single component took place at 20 000 r. p. m. At 50 000 r. p. m. further 4 components sedimented. The main component was of the lowest molecular weight. In *Protein IV*, 7 components were detected. Two of them sedimented at 20 000 r. p. m., the further 5 at 50 000 r. p. m. From *Protein V* at 20 000 r. p. m. 3 components sedimented while 1 component was floating at 50 000 r. p. m. and further 2 components sedimented.

As revealed by *Table I*, the protein fractions precipitated by ammonium sulphate were not homogeneous. In addition to the protein varying amounts of polysaccharide and nucleic acid were found to be present.

Composition of the protein was studied by amino acid chromatography.

Twelve different amino acids could be demonstrated in the hydrochloric acid hydrolysates. The amino acids were identified by parallel chromatography of known amino acids. The amino acid composition of all the 3 proteins obtained

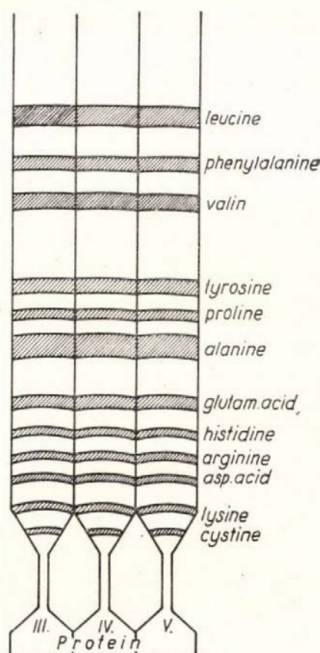


Fig. 3. Chromatogram of proteins hydrolysed by hydrochloric acid. Ninhydrin development

by fractionation with ammonium sulphate appeared to be identical. They contained leucine, phenylalanine, valine, tyrosine, proline, alanine, glutamic acid, histidine, arginine, aspartic acid, lysine and cystine. The chromatographic analysis of *Protein II* yielded similar results, with certain variations in the proportion of the different amino acids.

Our protein extracts contained also polysaccharides. Since the above results gave some information about their serological activity, further analysis of the polysaccharide components appeared to be important.

Chromatographic analysis of the sulphuric acid hydrolysates of our extracts revealed the presence of 3 carbohydrate components. On the basis of the diameter and colour intensity of the spots, these carbohydrates might be supposed to occur in different proportions in the different polysaccharides of the extracts. When identified by appropriate controls, the carbohydrate

spots appeared to correspond to those of arabinose, glucose and galactose. We must, however, mention that the chromatographic identification of glucose was not quite reliable since its epimer, mannose, has approximately the same R_f value.

In the preceding papers of this series [9, 11] we have reported on the isolation of polysaccharides from tubercle bacilli and we proved the cell wall origin of both the extracts *P I* and *P II*. On the contrary, neither α,ϵ -diamino-pymelic acid, nor muramic acid were present in our protein preparations, thus their cell wall origin could be excluded. We suppose that our com-

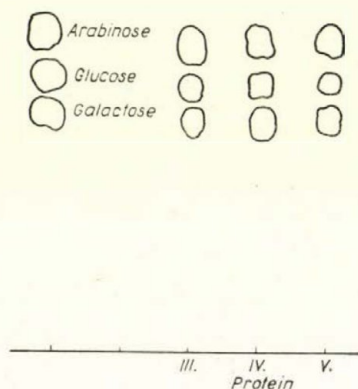


Fig. 4. Chromatogram of proteins hydrolysed by sulphuric acid.
Aniline-hydrogen-phthalate development

plex protein-like substances isolated may be the components of the internal protoplasmic material. Their place in our earlier schematic presentation [9] is somewhere in the middle.

Discussion

It was discovered by KOCH [24] that the protein-like substances in the bacillus were responsible for the tuberculin reaction. This very important observation has been corroborated by several authors. Because of this important biological property attention has been called to the TB proteins. Their isolation in chemically pure and biologically active form was the main goal of most of the studies. No experiments were made to elucidate their intracellular place, and data concerning their finer structure are also rather scarce.

HEIDELBERGER and MENZEL [1] extracted the bacterial cells with a buffer gradient from pH 4.0 to pH 11.0. The materials obtained differed both chemically and serologically. A minimum of 3 different antigens were found in the different fractions [25]. GRÖNWALL [2] disrupted the cells by freeze-drying and grinding. He further extracted this material with dilute alkali and separated its 3 components electrophoretically. One of them was a protein

precipitating at pH 5.5. WATSON and HECKLY [26] extracted the disrupted bacterial cells with an ether-phosphate buffer mixture [7] and with borate buffer. The extracts were filtered, dialysed and lyophilised. The nucleic acid was removed from the extract *in situ* by CaPO_4 . The material extracted with borate buffer contained more protein than that extracted by phosphate buffer. The protein preparation obtained by these authors proved to be a mixture of a number of different proteins.

A complex material was obtained by STACEY *et al.* [6] on treatment of the bacteria with a saturated solution of urea. This material was of high tuberculin activity and BALDWIN *et al.* [27] attempted to isolate its protein component. The material obtained, designated as UC protein, was found to be greatly homogeneous at electrophoresis. The amino-acid composition of fraction 101 B obtained by extraction with urea and that of the UC protein were examined by SEIBERT and FABRIZIO [28] and STACEY and LLOYD [29], respectively.

In our experiments the protein components of the substances isolated by fractionation of tubercle bacilli killed by heating to 100° C were found to be inhomogeneous. The extracts designated *Protein II* and *III* contained the highest amount of protein and comparatively the lowest of contaminants. The next two fractions, *Protein IV* and *V*, contained increasing amounts of nucleic acids and polysaccharides. The amount of polysaccharide varied between 20.3 and 41.5 per cent and that of DNA from 4.6 to 30.5 per cent.

Our extracts were serologically active. They precipitated the antibodies of sera obtained by immunizing rabbits by killed H₃₇ Rv strain cells. They exerted a haemagglutination inhibiting effect when appropriately tested. The quantitative relation in these serological reactions suggested that the serological specificity was associated with the polysaccharide components of these protein extracts. The results of double agar diffusion test revealed the serological homogeneity of our extracts, as only one precipitation boundary could be observed. The serological homogeneity did not exclude the possibility of a physico-chemical heterogeneity. In fact, our preparations exhibited a polydisperse molecular composition in ultracentrifugal examination. About 6 components of different molecular weight could have been separated. Nevertheless, this polydispersity should not be regarded as an absolute sign of heterogeneity as stated by TISELIUS [23]. Further electrophoretic analyses are needed to draw final conclusions.

A number of different amino acids were found in the proteins isolated from tubercle bacilli by SEIBERT and FABRIZIO [18] and STACEY and LLOYD [29]. As a result of these experiments, it seemed justified to conclude that the amino-acid content of tubercle bacilli is qualitatively stable, while certain quantitative variations might take place according to the methods of cultivation [30].

Alpha, ϵ -diamino-pymelic acid, characteristic of the cell wall was also found in tubercle bacilli [35, 32]. The protein fractions obtained in our experiments contained at least 12 amino-acids, while all were lacking both α , ϵ -diamino-pymelic acid and muramic acid. These observations suggested the supposition that these protein fractions did not originate from the cell wall. This supposition was further supported by the composition of the polysaccharide component.

The cell wall origin of the earlier isolated and examined polysaccharides *P I* and *P II* may be regarded as proven. A suggestion for their possible localization was also made [9]. On the basis of the methods of preparation and the analysis of the materials obtained we think that our protein substances contribute to the internal composition of protoplasm.

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Address of the author:

JÓZSEF FÖLDES

Institute of Microbiology, University Medical School, Beloiannisz tér 10, Szeged, Hungary.

LEPTOSPIROSIS STUDIES IN THE TÁPIÓ REGION

By

M. FÜZI and J. KISZEL

Institute of Microbiology, University Medical School, Budapest

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Summary. An outbreak of leptospirosis was observed in the village *Tápiószentmárton* and surroundings in the summer of 1954. Employees of the local farm and residents of the settlement were affected; the patients had bathed in the river *Tápió* or had worked in the fields. The clinical picture corresponded to mild leptospirosis; none of the cases was fatal in outcome. Of the 10 serologically verified cases 4 were caused by *L. pomona*, 4 by *L. grippityphosa* and 2 by *L. hyos*.

The sources of infection were studied by a serological screening of domestic animals in the summer of the following year (1955). Fifty pigs, 40 cattle and 42 sheep were examined by the agglutination-lysis test using the following serotypes: *L. icterohaemorrhagiae*, *L. canicola*, *L. ballum*, *L. australis* A, *L. australis* B, *L. pomona*, *L. grippityphosa*, *L. hebdomadis*, *L. sejroe*, *L. bataviae* and *L. hyos*. Of the pigs 42 (84 per cent), of the cattle 13 (32.5 per cent), of the sheep as few as 4 (9.5 per cent) proved to be positive. Pig sera reacted with the types *L. pomona*, *L. hyos* and *L. sejroe*: among the cattle infections with *L. pomona*, *L. sejroe* and *L. grippityphosa* were demonstrated; the 4 sheep sera showed low titres to *L. icterohaemorrhagiae*.

The epidemiological observations and the laboratory findings indicate, in spite of the simultaneous occurrence of the cases, the diversity of the sources of infection (pigs, cattle and field rodents).

Human leptospiroses were demonstrated in every region of Hungary during the last few years, but the incidence in the region between the rivers Danube and Tisza (Central Hungary) seemed to be rather low up to the year 1954. Few sporadic *L. pomona* infections had been found [1, 2]. In 1954, however, an epidemic was observed in the region of the river *Tápió* and the presence of 3 different leptospira serotypes was demonstrated in human infections. The present report deals with the epidemiological background of this particular epidemic.

In the summer of 1954, over a short period a number of leptospirosis-like cases occurred in the village *Tápiószentmárton* and surroundings. Twenty-two cases were observed between June 28 and July 14. The majority of the patients showed the symptoms of mild, non-icteric leptospirosis; in a few cases the course of the disease was more severe, but the outcome was never fatal. To verify the diagnosis of leptospirosis, serological tests were made, as at the start of the examinations the most favourable period for the haemoculture had been over. The blood specimens of ten patients were tested by the agglutination-lysis reaction. In each case paired sera were tested; the first sample was taken at the end of the first week after onset, the second sample during the

convalescence. The following serotypes were used in the tests: *L. icterohaemorrhagiae*, *L. canicola*, *L. ballum*, *L. australis A*, *L. australis B*, *L. grippotyphosa*, *L. hebdomadis*, *L. sejroe*, *L. pomona* and *L. hyos (mitis)*. The serological examination verified the diagnosis of leptospirosis in each of the 10 cases. Leptospiral antibody was not found in any of the acute samples whereas all the convalescent sera showed high titres, 4 against *L. pomona*, 4 against *L. grippotyphosa* and 2 against *L. hyos* (Table I).

Table I
Data of patients and results of serological tests (Tápiószentmárton, 1954)

No.	Initials	Age in years	Occupation	Onset	Acute serum		Convalescent serum	
					date	result	date	result
1.	D. B.	32	tiller	July 4	July 10	negative	July 29	<i>L. grippotyphosa</i> 1:1600
2.	J. Kh.	26	tiller	July 5	July 11	negative	July 29	<i>L. grippotyphosa</i> 1:800
3.	Is. Á.	15	tiller	July 5	July 11	negative	July 30	<i>L. hyos</i> 1:1600
4.	J. F.	15	farm hand	July 5	July 11	negative	July 29	<i>L. pomona</i> 1:800
5.	Im. Á.	52	tiller	July 6	July 12	negative	July 29	<i>L. grippotyphosa</i> 1:400
6.	S. D.	32	tiller	July 6	July 12	negative	July 29	<i>L. grippotyphosa</i> 1:200
7.	J. Ka.	34	farm hand	July 6	July 12	negative	July 29	<i>L. hyos</i> 1:800
8.	S. B.	18	farm hand	July 7	July 12	negative	July 30	<i>L. pomona</i> 1:400
9.	Is. Sz.	28	farm hand	July 7	July 12	negative	July 29	<i>L. pomona</i> 1:800
10.	J. B.	24	teamster	July 8	July 12	negative	July 29	<i>L. pomona</i> 1:800

Of these serotypes *L. pomona* had already been recognized in Central Hungary [1, 2], but the occurrence of the other types was surprising. *L. grippotyphosa* had rarely been observed in Hungary except in the western parts of the country, and *L. hyos* had been demonstrated only in North Hungary immediately before the epidemic under study [1, 2, 3]. The occurrence of three types in a circumscribed local outbreak was also surprising, so it was of interest to study the epidemiological aspects in the field.

Tápiószentmárton is a typical agricultural settlement, situated in the northern part of the area between the rivers *Danube* and *Tisza*, beside the river *Tápió*. It is surrounded by plough-lands, vineyards, fruit-gardens and pastures belonging partly to the community, partly to a neighbouring farm. Keeping domestic animals is very common in the village, and the live-stock of the farm (pigs, cattles, sheep and horses) is also considerable.

According to the data collected at the environmental survey, the epidemic was composed of at least three neighbouring focal outbreaks, in spite of the nearly simultaneous incidence of the cases.

The first suspected cases occurred between June 28 and 30 among children living in the village. The children had often bathed in the river *Tápió* on warm summer days. The small river, before arriving the village, crosses pastures, then its bed widens under a bridge; here the shallow water had been frequently used for bathing by a number of children of whom five fell ill. However, these cases were not examined by laboratory tests, thus their leptospiral origin is assumed on the basis of epidemiological features and the clinical pattern.

The second group of the patients consisted of labourers at the farm. Here 10 cases occurred between July 5 and 14. On the basis of the serological tests performed with the sera of 5 patients, *L. pomona* was the pathogenic agent in 4 cases and one additional case was caused by *L. hyos*. The teamster of the farm and a dairyman was among the patients, but most of them had worked in the large fruit-gardens, spraying vines. Considering the serotypes of the pathogenic agents it seemed likely that these infections had originated in domestic animals, and this was supported by the presence of a great number of domestic animals in the farm. On this basis the infection of the teamster and of the dairyman was readily explainable, but the workers employed at the fruit-gardens and vineyards had not been exposed to infectious materials of the domestic animals at their working place. However, the farm hands often bathed in the river in vicinity of the area where the pigs, cattle and sheep of the farm were pasturing during the summer months. Thus the farm hands could be infected by the contaminated water of the river or while staying on, or crossing, the pastures.

The third group of the patients was formed by labourers who had worked in a field around the farm *Alsótanya*, at a distance of about 8 km from the village. This place lies far away from the river, which had not been used for bathing by this group of patients. A total of 7 cases occurred here between July 4 and 9. Of the 5 cases tested serologically 4 proved to be caused by *L. grippityphosa* and one by *L. hyos*. All the patients were mowing and collecting hay during the incubation period, sometimes walking in ankle deep water as from hard rains during the previous weeks the ground was sodden. All the patients infected by *L. grippityphosa* had collected hay and worked at

the same place in the same group while the only *L. hyos* patient had worked at other places and had not stayed around the farm *Alsótanya*, near the group infected by *L. grippotyphosa*. Regarding the probable mechanism of infection, as the source field rodents were suspected in the *L. grippotyphosa* cases. These infections showed the typical pattern of "mud fever" which, according to European observations, is usually spread by field rodents [4, 5, 6]. Furthermore, rodents have proven to be common carriers of *L. grippotyphosa* in other parts of Hungary. Another possibility in these cases was to take into account the role of cattle in the transmission. According to recent reports [7, 8], cattle may be the chief reservoir of human *L. grippotyphosa* infections in some parts of the world. It should be noted that at the time of the outbreak 50–60 cattle were kept around *Alsótanya*. In the case of the *L. hyos* infection, on the other hand, the role of domestic animals had to be supposed, these being everywhere responsible for the spreading of *L. hyos* [6, 9]. Thus in this single case the cattle around *Alsótanya* might have been the source of infection. It cannot be excluded, however, that the patient had been infected at some other place where he was doing occasional work.

On the basis of the above data the leptospirosis outbreak at *Tápiószentmárton* can be regarded as composed by sporadic cases and focal outbreaks which might have originated in several neighbouring sources, and the mode of transmission might have also been different. As the incidence of leptospiral infection among the animals in this region was unknown, it was decided to make a survey of the farm animals kept at the pastures along the river *Tápió*, where the majority of the human cases had occurred.

Of the live-stock of the farm 50 pigs, 40 cattle and 42 sheep were examined with the agglutination-lysis test in August, 1955, using the following serotypes: *L. icterohaemorrhagiae*, *L. canicola*, *L. ballum*, *L. australis* A, *L. australis* B, *L. pomona*, *L. grippotyphosa*, *L. hebdomadis*, *L. sejroe*, *L. bataviae* and *L. hyos*. Titres of 1:100 and above were regarded as positive. If a serum sample reacted with two or more unrelated serotypes, the animal was regarded as double- or multiple-infected; double and multiple reactions with related serotypes, on the other hand, were considered as co-agglutinations [see also 3, 10, 11]. In the following we present the results of the serological survey, by animal species (*Table II*).

The infection rate among the pigs was rather high. Of the 50 animals (47 sows and 3 bears) 42 (84 per cent) had leptospiral antibodies in their sera. Positive results were obtained with the strains *L. hyos*, *L. pomona* and *L. sejroe*; with the other strains at most slight co-agglutination was observed. The majority of the sera [31] reacted with a single type; 10 sera reacted with 2 and one with all the three types mentioned. The infection rate was the highest for *L. hyos* (26 per 50), but that for *L. pomona* was also high (21 per 50). Positive reaction with *L. sejroe* was observed occasionally (7 cases). According to the

Table II

Distribution of seropositive domestic animals by leptospiral types (Tápiószentmárton, 1955)

		Number of		
		Pigs	Cattle	Sheep
Positive against	<i>L. hyos</i>	17	—	—
	<i>L. pomona</i>	12	8	—
	<i>L. sejroe</i>	2	2	—
	<i>L. hyos + pomona</i>	6	—	—
	<i>L. hyos + sejroe</i>	2	—	—
	<i>L. pomona + sejroe</i>	2	2	—
	<i>L. hyos + pomona + sejroe</i>	1	—	—
	<i>L. grippotyphosa</i>	—	1	—
	<i>L. icterohaemorrhagiae</i>	—	—	4
Total positive		42	13	4
Negative		8	27	38
Grand total		50	40	42

information supplied by the veterinarian of the farm, in spite of the high infection rate, no leptospirosis-like disease or frequent abortions occurred among the pigs during the previous three-year period.

The infection rate for the cattle was markedly lower. Of the 40 animals tested 13 (32.5 per cent) proved to be seropositive. Eleven sera reacted with a single serotype, two sera with two types. Reaction with *L. pomona* was the most frequent (10 cases), that was followed by *L. sejroe* (4 cases) and *L. grippotyphosa* (1 case). The age of the cattle tested varied between 3 and 9 years, and there was no significant fluctuation in the age distribution of positive results. Leptospirosis-like disease had not been observed among the cattle.

The serological survey of the sheep yielded negative result regarding the incidence of the serotypes found in the human infections. Of the 42 whethers and ewes as few as 4 gave titres, notably low titres (1:100), with *L. icterohaemorrhagiae*. These weak reactions might be considered as due to residual antibodies from previous *L. icterohaemorrhagiae* infections, or as co-agglutinations due to infections by a serotype not used in our investigations. Finally, since higher titres did not occur, a non-specific agglutination cannot be excluded either. It should be emphasized that none of the sera reacted with serotypes other than *L. icterohaemorrhagiae*.

The screening tests described above have provided evidence that the domestic animals kept along the river Tápió might have been of epidemiological importance in the human leptospirosis outbreak under study. The infection

rate among the animals was high, and mainly those serotypes (*L. pomona* and *L. hyos*) were demonstrable by which the human cases had been caused. However, the importance of the three species tested was different. The fact that the pigs were highly infected with both *L. hyos* and *L. pomona* suggests that the infective excretions of this species might have been the most probable sources of infection for humans. Nevertheless, some *L. pomona* infections might have originated in cattle, for this serotype was frequently present in the cattle, too. On the other hand, the serological results, in contrast to the epidemiological possibility are against the role of sheep in human infections.

Discussion

Human leptospiroses generally occur sporadically. Epidemics are rare, circumscribed small outbreaks are more frequent than extended epidemics [1, 2]. Leptospiral infection is rather widespread among different species of animals, but transmission to humans needs particularly favourable conditions, such as (i) the presence of a great number of sources of infection; (ii) humid milieu, in which leptospires can survive for a long time; (iii) a great number of susceptible persons at the infested site. In Hungary most of the epidemics have been observed (i) during the summer months; (ii) in rural milieu; in connection with (iii) collective bathing in surface waters; or (iv) with agricultural work carried out in groups [1, 2].

The epidemic under discussion occurred during the summer months and in rural milieu, but at a place where leptospirosis had never been observed; the outbreak was caused by various serotypes of leptospires, and the mode of transmission was also heterogeneous.

Unexpected appearance of leptospiroses, either sporadic or epidemic-like, in a region where they have never been observed does not indicate that leptospirosis has never occurred there. It is often quite difficult to recognize animal leptospiroses without laboratory examinations, and sporadic human cases may also escape attention. Leptospiroses of wild rodents are not indicated by observable epizootics, although in recent reports it has been suggested that the rodents' disease is not always mild; it is often associated with chronic nephritis [12]. According to our observations [3, 11], dogs may be infected at a high rate without showing any marked sign of illness. Among larger domestic animals a more serious course of the disease, abortion and fatal outcome are more frequent, but the occurrence of these signs greatly depends on secondary factors, viz. age, nutritional state, simultaneous occurrence of other diseases, pregnancy at the time of the infection, etc. [6, 8, 9, 14]. These points explain the lack of clinically-recognizable leptospiroses in an area in spite of the serological evidence of a high infection rate. A similar situation was revealed in

the case of the pigs and cattle involved in this study. A subsequent examination has suggested that leptospirosis was present not only in the farm, but also among the pigs kept in the village. Serum samples of two pigs belonging to individual households in the settlement gave 1:1600 and 1:3200 titres, respectively, to *L. sejroe*. It could, accordingly, be assumed that leptospirosis was not recently introduced into this region, but at the time of the outbreak conditions were especially favourable for the human infection. The large number of infected animals, the humid milieu and the rainy weather were the factors increasing the risk of infection for persons working in fields or bathing in surface waters.

Another notable characteristic of the *Tápiószentmárton* outbreak was the presence of different serotypes and of different modes of transmission in a local epidemic limited to a short period of time. For most of the human epidemics observed earlier one serotype was described as the pathogenic agent. The majority of the outbreaks is really caused by a single serotype, but, since the number of known serotypes has increased, the simultaneous occurrence of several serotypes has been observed with increasing frequency. We too have reported on an explosive outbreak caused by two serotypes, namely by *L. canicola* and *L. pomona* [13]. Such outbreaks are due to the local presence of an animal herd harbouring different leptospira types or to various animal species excreting a variety of serotypes. The aetiology and mode of transmission in such seemingly homogeneous outbreaks cannot be revealed but by laboratory investigations. It seems reasonable to suggest that in the present outbreak the disease was transmitted under favourable conditions by different animal species excreting different types of leptospires.

The results of the serological screening tests essentially agreed with our former observations made elsewhere in Hungary [3, 10]. Among pigs *L. pomona* and *L. hyos* are widely spread, and the presence of *L. sejroe* is also common. Among cattle, on the other hand, *L. pomona* and *L. sejroe* are more frequent while *L. hyos* was demonstrated only in single foci. The present results confirm our earlier observation [3, 10] that, unlike wild rodent populations, domestic animal herds may simultaneously be infected by several types of leptospires. The failure to demonstrate antibodies against the above serotypes in the sheep sera tested does not mean that this species is not susceptible. It is well-known that sheep can be infected by a number of leptospiral serotypes, and the leptospiral infections of the sheep simulate the serious disease of the cattle [14, 15, 16]. Our recent screening tests have provided serological evidence that leptospiral seropositivity is frequent among sheep elsewhere in Hungary. In the present case the lack of antibodies might be explained by the fact that in the farm the sheep were kept and pastured separate from the pigs and cattle.

Accordingly, mingling of different domestic animals (first of all the presence of pigs among them) is an important factor in the spreading of

leptospiroses. It is therefore desirable to keep the animals in small groups at a fair distance from each other. Special attention should be paid to the possibility of transmission by water, thus the infected live-stock, especially swine, should be maintained downstream from the other herds.

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Address of the authors:

MIKLÓS FÜZI

Institute of Microbiology, University Medical School, Hőgyes Endre u. 7—9, Budapest IX, Hungary.

JÁNOS KISZEL

2nd Department of Pediatrics, University Medical School, Tüzoltó u. 7, Budapest IX, Hungary.

STUDIES ON THE TECHNOLOGY OF PREPARATION OF GLUCOSE-CONTAINING MEDIA

MASS PRODUCTION OF RHIZOBIA

By

B. JOHAN, J. SZABÓ SZÜCS and ARANKA SZABÓ

Institute of Serum and Vaccine Production "Phylaxia", Budapest

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Summary. For the rapid starting of growth of *Rhizobium meliloti* a stimulatory substance is required which is formed in glucose-containing media during sterilization by heat. When on the other side the influence of heat is performed beyond a certain limit, the lag phase becomes longer or occasionally growth does not start at all. Exposure to a higher temperature after a certain period results in a brown coloration of the described glucose-containing media. The browning of these media can be observed after a longer storage at room temperature as well (Maillard's reaction). The intensity of the brown colour is proportional to the degree of heating and to the length of exposure to it. By the use of a photometer and an adequate colour filtre, the intensity of the colour can be expressed as the extinction index. The increase of the extinction index is accompanied by the prolongation of the lag phase. No *Rhizobium* growth occurs in media whose extinction index exceeds a certain value. The method is applicable as a control of the sterilization procedure of glucose-containing media in fermentors.

In order to produce vitamin B₁₂ and cultures for the inoculation of soil, *Rhizobium* was grown in shake cultures and later in fermentors. The culture medium consisted of natural organic materials, glucose and salt. The medium containing all the ingredients was sterilized in an autoclave at 120° C for 20 to 30 minutes. *Rhizobium* strains generally showed a microscopically well-detectable multiplication in this medium after 5 to 6 hours. Subsequently growth rapidly increased and the maximum density was reached between the 28th and 32nd hour. Sometimes the rapid growth started only in the 20th to 40th hour of incubation, or, occasionally no growth occurred even after this period. In the latter case the strain, medium, ingredients, pH, etc. were the same as those employed when satisfactory growth was obtained. This suggested some undetected differences in the technology of the medium's preparation.

When preparing liquid media for the cultivation of microorganisms it is generally recommended that sugars should be added to the media only after the separate autoclaving of the latter. The sugars (possibly together with the phosphates) should be dissolved in a small amount of water and autoclaved separately or made free from germs by filtration (*e.g.* through Seitz or glass filters). It has been, namely, found that certain microorganisms grew poorly or not at all when the sugar had been added to the medium prior to sterilization. Therefore shake culture experiments were performed and the growth of *Rhizobium* was examined in a medium to which separately sterilized sugar and phosphate had been added.

In these experiments, however, growth was not so abundant and the production of vitamin B₁₂ was less successful than in media whose ingredients had been autoclaved together. (Leaving by side now the cases mentioned in the first paragraph.) Besides, in large-scale production the sterilization of the ingredients in two batches is inconvenient. The purpose of the present work was therefore to find the most practical solution of the problem.

Materials and methods

Test cultures. In the experiments 67 different *Rhizobium* strains were used. Most of the examinations were performed with strain *Rhizobium meliloti* No. 760, kindly supplied by A. G. Lockhead (Ottawa, Canada). In this laboratory the strain was designated as Rh. 66. The strain was maintained on lucerne extract agar.

Medium for the preservation of cultures. Powdered lucerne 3.0 g, sucrose 20.0 g, K₂HPO₄ 0.2 g, MgSO₄ · 7H₂O 0.2 g, agar 20.0 g, distilled water 1.0 l; pH 7.0. The powdered lucerne was boiled in 250 ml of water for 15 minutes then filtered and the clear liquid was added to the solution of the other ingredients prepared with the remaining 750 ml of water.

Culture media. The inocula and sometimes the cultures themselves were grown in medium "L": Casein hydrolysed with acid 15.0 g, baker's yeast extract (dry weight) 4.0 g, glucose 30.0 g, K₂HPO₄ 0.5 g, MgSO₄ · 7H₂O 0.5 g, KCl 0.2 g, FeSO₄ · 7H₂O 0.02 g, CoCl₂ · 6H₂O 0.012 g, tap water to make 1.0 l solution; pH adjusted before autoclaving to 7.8. After sterilization the pH was 6.6 to 6.8.

For fermentations medium "LX" was generally employed: Corn steep liquor (dry weight) 24.0 g, brewer's yeast autolysate (total nitrogen) 354.0 mg, glucose 20.0 g, KH₂PO₄—KOH stock solution 10.0 ml, MgSO₄ · 7H₂O 0.5 g, KCl 0.2 g, FeSO₄ · 7H₂O 0.02 g, CoCl₂ · 6H₂O 0.012 g, tap water to make 1.0 l solution; the pH was adjusted with NaOH before autoclaving to 6.6. Sterilization of the medium was at 120° C in shake culture flasks for 20 minutes, in fermentors for 30 minutes or sometimes for 20 minutes (KH₂PO₄—KOH stock solution: KH₂PO₄ 8.8 g, KOH 1.28 g, distilled water to make 100 ml.).

Preparation of inocula. The growth from a 24 hour lucerne agar slant was suspended in 5.0 ml of distilled water. One half ml portions of this suspension were transferred into 500 ml Erlenmeyer flasks each containing 100 ml of medium. The inoculated flasks were shaken on a plansifter adapted for this purpose for 48 hours at 26° C. Each of the flasks made a circle 30 mm in diameter at 320 r. p. m.

Inoculation of shake flasks and fermentors. As a rule 2 per cent of inoculum was used. The shake culture experiments were carried out in at least two parallel flasks. Cultivation was performed at 26° C. JOHAN—LOVREKOVICH glass fermentors were supplied with 0.5 l air per 1.0 l medium per minute. Two 2 paddled stirrers operating at 500 r. p. m. were arranged to run one over the other. As an antifoam agent sunflower oil was used. Stainless steel fermentors of 20 to 100 l capacity were equipped with turbostirrers.

Estimation of bacterial growth. Turbidity standard: To 9.6 ml 1 per cent sulphuric acid 0.4 ml 1 per cent barium chloride was added and the mixture was placed into a Jena-glass test tube and stoppered airtightly. Into a similar tube 1.0 ml of the examined culture was pipetted and the suspension was diluted until its turbidity corresponded to the standard. The suspensions were examined in a comparator or with a nephelometer. *Rhizobium* growth (density) was expressed as the rate of dilution. One degree of dilution corresponded to approximately 10⁹ cells per ml. The turbidity standard could be used for two weeks.

Estimation of vitamin B₁₂ was performed by the E. coli-test described by HARRISON, LEES and WOOD [1].

Colorimetric examination of the medium. The medium was filtered to perfect clarity and the extinction was determined against water by means of a Pulfrich photometer and an S₃₀ filtre. The extinction obtained this way was called the "extinction index".

Experimental

(1) *Rhizobium* growth and vitamin B₁₂ production were examined in shake cultures at different pH values in two types of medium "L". One of the media was autoclaved together with the sugar, the other prior to the addition of the separately sterilized sugar (Table I).

Table I
Rhizobium growth and vitamin B₁₂ production
(Medium autoclaved at different pH values; sugar sterilized incorporated in the medium or separately)

Designation of culture	pH prior to sterilization	Mode of sugar sterilization	Growth of <i>Rhizobium</i>	B ₁₂ production $\mu\text{g/ml}$
30/5	7.2	incorporated	24	3.70
30/6	7.2	separately	18	3.14
30/7	7.8	incorporated	20	4.87
30/8	7.8	separately	21	2.82

In the medium autoclaved at pH 7.2, growth and production were better when the sugar had been added before sterilization.

In the two types of media which had been autoclaved at pH 7.8 the bacterial count showed but a slight difference; production of vitamin B₁₂ was, however, copious in the medium sterilized together with the sugar.

(2) The influence of the time of sterilization on the growth and vitamin B₁₂ production by the *Rhizobium* was examined in shake cultures. In these experiments strain Rh 66/27, a less mucous variant of strain Rh 66 was used (Table II).

Table II
Rhizobium growth and vitamin B₁₂ production in media autoclaved for various times

Designation of culture	Medium	Length of sterilization, minutes	Growth of <i>Rhizobium</i> at hour-			Peak value of B ₁₂ $\mu\text{g/ml}$
			24	48	72	
69/1	LX	10	13	24	38	2.30
69/2	LX	20	14	26	39	3.10
69/3	LX	40	14	32	38	3.90
69/4	LX	60	2	12	39	3.20
69/17*	L	10	1	24	38	—
69/18*	L	20	1	26	36	—
69/19*	L	40	0.5	26	38	—
69/20*	L	60	—	2.5	38	—

* Inoculated directly with suspension obtained from lucerne extract agar.

In the medium autoclaved for 20 minutes growth was somewhat better and the peak production of vitamin B₁₂ definitely higher than in the medium sterilized for 10 minutes. In media autoclaved for 60 minutes the lag phase was long; multiplication started only after 24 (occasionally after 48) hours. However, after this delayed start, rapid multiplication ensued and by the 72nd hour this culture reached a peak equivalent to that of the others.

(3) Although these shake flask experiments were not repeated in the 5 litre fermentors, it was often observed that in the latter growth started only after a prolonged lag phase (20 to 46 hours) or not at all. On one occasion two fermentors with the same media were sterilized at 121° C for 30 minutes. As soon as the autoclave cooled to about 90° C one of the fermentors was removed to room temperature. The second fermentor remained in the autoclave until next morning (for about 12 hours), and so let to cool slowly. As a result of the prolonged cooling, the medium in this second fermentor was exposed to a heat ranging from 90° to 25° C for a considerably longer period than the medium in the other fermentor (*Table III*). In the rapidly cooled fermentor the lag phase was somewhat longer than usual (sterilization at 120° C for 30 minutes was too much); in the medium which had been left to cool in the autoclave growth practically did not start.

Table III

Growth of Rhizobium in fermentors cooled rapidly, resp. slowly, after autoclaving

Designation of culture	Cooling of fermentor	Growth of Rhizobium at hours				
		0	16	24	40	48
63/I	rapid	0	1	7	22	22
63/II	slow	0	0	1	1	1

(4) During cultivation difficulties were met with particularly when the procedure was carried out in 20 and 100 then in 600 and 1000 litre metal fermentors. Parallel cultures with the same batch of medium in shake flasks and 5 litre glass fermentors which had been sterilized in the autoclave, yielded a good growth, whereas in the large fermentors the growth started more than once 20 to 30 hours late or not at all. The difference was striking as *Rhizobium* was not sensitive to iron (it grew well in media containing ferrous ions up to 200 µg/ml) and, in addition, the fermentors were made of stainless steel. All the containers from the shake flasks to the large fermentors were sterilized at 121° C for 20 minutes. There was, however, a difference in the time necessary for heating the media from room temperature to 121° C and for cooling them again to room temperature. With shake flasks this procedure lasted only for 2 to 3 hours, while the large fermentors required at least 4 to 5 hours. Accord-

ingly, the medium in the latter was exposed to high temperature for a considerably longer period than medium autoclaved in small containers.

(5) Observing that the brown colour of the medium in fermentors yielding no growth was distinctly darker than the colour in other fermentors, the intensity of the colour was determined by means of a Pulfrich photometer. The results obtained with medium "LX" are presented in *Table IV*.

Table IV
Extinction index of media sterilized in various containers

	Exposure to 121° C, minutes	Extinction index
Medium prior to sterilization	0	0.20—0.23
Medium sterilized in shake flasks	20	0.35—0.45
Medium sterilized in 5 l glass fermentors	30	0.42—0.50
Medium sterilized in 100 l metal fermentors	20	0.47
Medium sterilized in 100 l metal fermentors	30	0.83
Medium sterilized in 100 l metal fermentors	60	1.25

(6) A comparison of the growth of *Rhizobium* in media with different extinction indices is presented in *Table V*.

Table V
*Connection between *Rhizobium* growth and the medium's extinction index*

Growth of <i>Rhizobium</i>	Extinction index
Optimum	0.40—0.50
Scanty after a long lag phase	0.80—1.00
Exceptional	> 1.20

(7) Shake flask cultures with freshly prepared medium "L" yielded good and rapid growth.

When storing the medium at room temperature, growth became gradually poorer and finally did not start at all. The connection between this finding and the extinction index of the medium is set out in *Table VI*.

(8) In subsequent experiments various amounts of *Rhizobium* were inoculated into shake flasks which had been sterilized at 121° C for various periods. The inoculum of flasks "a" contained 23 times as many bacteria as that of flasks "c".

Table VI

Connection between Rhizobium growth and extinction index of medium stored at room temperature

Culture medium	Extinction index	Growth of Rhizobium
Freshly prepared	0.40	Optimum
Stored for 3 months	0.85	Scanty after a long lag phase
Stored for 5 months	1.50	None

Table VII shows that when a large inoculum was used, in the flasks sterilized for short periods (20 to 30 minutes) the maximum growth was reached at the 48th hour. In the flasks sterilized for longer periods (50 to 60 minutes) the peak of growth was attained by the 72nd hour. At this time a slight degree of autolysis occurred in the former cultures. As to the cultures inoculated with less bacteria, a striking difference was found among the flasks sterilized for various periods. In the medium autoclaved for more than 30 minutes growth hardly occurred.

Table VII

Growth of Rhizobium in shake flasks sterilized for various times and inoculated with different amounts of bacteria

Designation of culture	Time of autoclaving minutes	Inoculum $\times 10^9$	Growth of Rhizobium at hours		
			24	48	72
1a	10	28	12	30	30
2a	20	28	8	38	30
3a	30	28	8	34	30
4a	40	28	8	32	32
5a	50	28	8	26	36
6a	60	28	6	12	32
1c	10	1.2	4	20	30
2c	20	1.2	6	22	30
3c	30	1.2	.	10	30
4c	40	1.2	.	6	6
5c	50	1.2	4	4	4
6c	60	1.2	2	4	4

Discussion

From the above observations two conclusions may be drawn.

One that the *Rhizobium meliloti* strain used grows better in glucose-containing medium and produces an increased quantity of vitamin B₁₂ when the sugar is autoclaved together with the medium.

This behaviour of *Rhizobium* is not a unique phenomenon in microbiology. RAMSAY and LANKFORD [2] observed that *Saccharomyces cerevisiae* cultures require a stimulatory substance which is formed when sugar-containing media are subjected to heating. A similar growth-promoting factor was found necessary for the cultivation of *Streptococcus faecalis*, *Str. lactis*, *Lactobacillus leichmanii*, *L. delbrücki*, *L. arabinosus*, *L. fermenti*, *Micrococcus pyogenes aureus* and *Leuconostoc mesenteroides*. No such stimulatory effect was needed by cultures of a number of other bacteria as *Pseudomonadaceae*, *E. coli* and *S. typhi*. The shortening of the lag phase by a substance produced on heating was observed and investigated in detail by FIELD and LICHSTEIN [3] in propionic acid bacteria and by SERGEANT, LANKFORD and TRAXLER [4] in *B. globigii*. It was shown that *B. cereus*, *B. subtilis*, *B. megaterium* also showed increased growth if the medium had been sterilized together with the sugar. The substance shortening the lag phase of bacteria in autoclaved glucose-containing media was named by SERGEANT *et al.* the "glucose phosphate factor". These authors extracted and concentrated this substance and added it to a sugar-medium which had been sterilized by filtration. This medium exhibited a stimulatory action on *B. globigii*.

With *Rhizobium* there was no need to isolate the stimulatory substance ("glucose phosphate factor"); it was provided by the simultaneous sterilization of all the ingredients of the media. The disadvantage of the simultaneous sterilization, however, had to be eliminated.

The second conclusion drawn from the present observations and experiments is as follows.

When in order to assure sterility, the time of sterilization is prolonged, a certain point is easily reached beyond which the growth of *Rhizobium* will start late, and when heating is further lengthened, the bacteria will fail to multiply. Thus the aim of obtaining rapid and optimum growth is often hindered by the endeavour of assuring sterility.

The inhibitory action following the sterilization of glucose-containing media by heat has been observed on various bacteria. Such an effect was shown by LEWIS [5] on *Phytomonas malvaceara* isolated from cotton, by HILL and PATTON [6] on *Streptococcus faecalis* and by FINKELSTEIN and LANKFORD [7] on *Vibrio cholerae*, *S. paratyphi*, *E. coli* and *Serratia marcescens*. *Ps. aeruginosa* and *S. typhi* *murium* were not sensitive to the inhibitory action. The hypothesis that a toxic substance is formed on autoclaving such media has been rejected and preference is given to the explanation put forward by LEWIS and others. In their view in the presence of phosphates, glucose dissociates on heating. The aldehyde produced forms a complex with the nitrogen-containing ingredients of the medium. This complex compound is utilized by certain bacteria, but not by others. The reaction was originally described by MAILLARD [8] in 1912. That the bond of glucose and amino acids results in

amino acid deficiency was shown in cysteine-requiring gonococci by LANKFORD, RAVEL and RAMSAY [10] and in tryptophan-requiring *Str. faecalis* by HILL and PATTON [11]. According to FINKELSTEIN and LANKFORD, multiplication may occur in "over-sterilized" media provided that the organism is able to produce an adaptive enzyme which breaks down the complex compound. Such an observation was made when *Rhizobium* still started to grow after a prolonged lag phase in media which had not been heated beyond a certain period of time.

Media exhibiting a definite inhibitory action were rather dark brown in colour. The brown colour was moderate when glucose had been sterilized for a longer period in the presence of phosphate only. If, however, the protein-containing components were incorporated, the brown colour of the medium became much more intense. According to MAILLARD during this process "melanoidines" are produced. The brown substance is not identical with the yellowish-brown one formed on caramelization. We found *Rhizobium* to be able to utilize glucose caramelized at 180°C as well as the non-caramelized sugar. Therefore the dark brown colour of autoclaved glucose-containing media can be considered as a *Maillard*-type reaction.

The present investigations have shown that with the usual sterilization procedure at 120° C the degree of browning is proportional to the whole time of exposure to heat. The reaction also takes place in our sugar-media stored at room temperature, but, of course, much more slowly than on heating. The intensity of browning at room temperature is proportional to the length of storage.

The intensity of the brown colour was determined by means of a Pulfrich photometer and was expressed as the extinction index. The higher the extinction index the longer was the lag phase of growth of *Rhizobium*. Beyond a certain value growth did not start. This finding shows that when glucose-containing media for *Rhizobia* are to be sterilized care should be taken that the extinction index does not exceed 0.60. Over this value the lag phase may possibly be shortened by the use of more abundant inoculum. With an extinction index over 1.0 there is no much hope of obtaining growth even with large inocula.

The determination of the extinction index helps in the elaboration of the sterilization technique of glucose-containing media for large fermentors. First the extinction index of the medium yielding optimal growth should be determined. Then the heating and particularly the rate of cooling of the fermentor following sterilization should be so regulated that the extinction index does not exceed the value required. If a moderately higher value has been reached, the above-mentioned method of inoculation will possibly be helpful.

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Address of the authors:

BÉLA JOHAN
Kelenhegyi út 33, Budapest XI., Hungary.

JÁNOS SZABÓ SZÜCS, ARANKA SZABÓ
Institute of Serum and Vaccine Production "Phylaxia", Szállás u. 5—7, Budapest X., Hungary.

Addendum

Since our paper was sent in for publication, we have read E. L. GADEN's report (*Appl. Microbiol.* **8**, 123, 1960), in which it is pointed out that even a nominally uniform sterilization procedure (*e. g.* at 120° C for 15 minutes) may act differently on the medium, and that the effect depends on the time required for heating and cooling of the medium in the apparatus used. According to this author the "total heat input" determines the alterations taking place in the medium. Our experiments described in the above paper led us to the same conclusion. In addition, we worked out a method for the determination of the changes in media containing protein, phosphate and sugar. By this method the effect of the total heat input, which had been hypothetical before, can be properly determined and the uniformity of the chemical changes taking place in the medium during heat sterilization can be ensured.

A COMPARATIVE STUDY OF THE EFFECTIVENESS OF DIFFERENT STANDARD PERTUSSIS VACCINES

By

I. JOÓ, SUSANNA PUSZTAI and VERA P. JUHÁSZ

Institute for Serobacteriological Production and Research "Human", Budapest

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Summary. Standard pertussis vaccines prepared in this laboratory were compared by the active mouse protection method to standard vaccines from abroad.

No significant difference was found between the American (USV), the British (BV) and the Hungarian (Hu V 1) standard vaccine preparations.

Both Hu V 2 and Hu V 3 standards prepared in our laboratory appeared to be more effective (about one and a half times) than the USV, BV and Hu V 1 standard vaccines. The difference was not statistically significant.

The protective value of the international standard vaccine (IV) was significantly higher than that of the other standard vaccines.

No significant differences were found between the individual results of parallel determinations of the protective value of the same vaccine within the same experiment.

Certain problems of the evaluation of the results obtained by active mouse protection test are discussed.

An important progress in the field of pertussis vaccination has recently been achieved by the field trials organized by the Medical Research Council in England [1—3]. These trials yielded information partly about the protective value of different vaccine types, partly about the correlations between the epidemiological effectiveness and the results of laboratory testing of the same preparations. The discussion and evaluation of these results on a very broad basis made the development of standard pertussis vaccines possible, first in the United States [4] later also in England [5] and the Netherlands [6]. This was soon followed by the preparation and acception of an international standard pertussis vaccine [7]. The standards were prepared partly from vaccines of reliable protective activity in humans, partly from preparations which had proved identical to the former in careful laboratory examinations. Thus these were definitely acceptable for estimating the immunogenicity of routinely prepared pertussis vaccines. As the active mouse protection test appeared to give results best characterizing the actual human protective value, this test was used for the control of pertussis vaccines.

In the present paper we report on studies followed since 1954, the preparation of our own standard vaccines and their comparison to some foreign standards.

Materials and methods

Vaccines: U.S.A. standard vaccine (USV), National Institutes of Health, Bethesda, Maryland, batch N° 5. From this material 4 samples were received since 1954. They were designated USV I, II, III and IV. Each ml of vaccine contained 96×10^9 germs. The samples were treated as prescribed and they were generally used for 1 year following resuspension.

Lyophilized British standard vaccine (BV). This was obtained from the National Institute for Medical Research, London. Each ampoule contained 50×10^9 germs. The ampoules were opened immediately before use and suspended in 1 ml distilled water.

Lyophilized international standard vaccine (IV), Statens Seruminstitut, Copenhagen. Each ampoule contained 50×10^9 germs. The ampoules were opened immediately before use and suspended in 1 ml distilled water.

Own lyophilized standard vaccine N° 1 (Hu V 1). The vaccine was prepared in November, 1954, from a 3 days old Bordet-Gengou medium culture of *B. pertussis* 41405. The cells were killed by merthiolate 1 to 5000. The stock suspension was diluted so as to contain 60×10^9 germs per 0.2 ml (according to the NIH opacity standard). The vaccine was filled in ampoules (0.2 ml each) and lyophilized. The material was stored at 4° C until used.

Own lyophilized standard vaccine N° 2 (Hu V 2). This was prepared from the stock suspension 546/3 prepared in June, 1956. This was an equal mixture of strains 41405, 324 E, 358 E, CN 2894, CN 2896 and CN 2897. The cells were obtained by cultivating for 3 days on a solid semi-synthetic medium containing 0.3 per cent active coal [8, 9], and killed with merthiolate 1 to 5000. The standard vaccine was prepared from this suspension in June, 1958. Three dilutions were prepared in saline containing 6 per cent dextran and merthiolate 1 to 10 000. The dilutions contained 45×10^9 (2/1), 67.5×10^9 (2/2) and 90×10^9 (2/3) germs per ml, respectively. One ml samples of the dilutions were filled in ampoules and lyophilized. The ampoules were stored at 4° C until used.

Own lyophilized standard vaccine N° 3 (Hu V 3). This was prepared in August, 1958, from the stock suspension 546/3 in a way essentially similar to that described above. The standard vaccine contained 90×10^9 germs per ml. This suspension was ampouled in 1 ml volumes and lyophilized. The material was stored at 4° C until used.

Samples of our own three standard vaccine preparations were opened immediately before use.

Active mouse protection test. This was performed according to the NIH prescription [10]. Sixteen mice each were vaccinated with 3 different doses of vaccine. The animals were challenged after 16 days by intracerebral inoculation of 100 000 germs of N° 18 323 strain of *B. pertussis*. The animals were observed for a fortnight. The results were evaluated statistically by the WILSON—WORCESTER method [11].

ED_{50} = 50 per cent protective dose as expressed in million germs;

1 S.D. = logarithm of the standard deviation of ED_{50} ;

α = standard deviation and logarithm of homogeneity factor of titrations.

The difference between two vaccines was significant if

$$\log ED_{50} (St) - \log ED_{50} (X) > 2 \text{ S.D.}$$

where

$$2 \text{ S.D.} = 2 \sqrt{[S.D. ED_{50} (St)]^2 + [S.D. ED_{50} (X)]^2}$$

St = standard vaccine;

X = vaccine examined

The relative potency (RP) of the vaccines was calculated from the ratio of ED_{50} values of the standard and test vaccines. In these calculations the standard vaccine was taken as unit.

Results

Comparison of standard vaccines Hu V I, USV I and II. As determined in preliminary experiments the following immunizing doses were used in our experiments: 3000×10^6 , 600×10^6 , 120×10^6 germs per dose of USV I and II, and 500×10^6 , 100×10^6 and 20×10^6 germs per dose of Hu V 1 vaccine. Results are presented in Table I.

Table I
Comparison of vaccine preparations Hu V 1 and USV I, II

N ^o of experiment	Date		Hu V 1	USV I	USV II	LD ₅₀ ± S.D.	Significance
			1	2	3	4	5
1.	1. VI. 1955.	ED ₅₀ IS.D. α RP	56.75 ± log 0.151 log 0.814 ± 0.219 7.12	598 ± log 0.145 log 1.210 ± 0.350 0.67	404 ± log 0.180 log 0.930 ± 0.312 1.0	2385 ± log 0.368	1 : 2 > 2S.D. 1 : 3 > 2S.D. 2 : 3 < 2S.D.
2.	13. VI.	ED ₅₀ IS.D. α RP	251 ± log 0.302 log 0.602 ± 0.238 3.36		845 ± log 0.129 log 1.433 ± 0.395 1.0	851 ± log 0.231	1 : 3 < 2S.D.
3.	13. VIII.	ED ₅₀ IS.D. α RP	154.5 ± log 0.128 log 1.472 ± 0.404 7.85	1071 ± log 0.205 log 0.812 ± 0.301 1.14	1218 ± log 0.208 log 0.815 ± 0.301 1.0	2275 ± log 0.155	1 : 2 > 2S.D. 1 : 3 > 2S.D. 2 : 3 < 2S.D.
4.	14. IX.	ED ₅₀ IS.D. α RP	a) 178.5 ± log 0.206 log 0.816 ± 0.298 2.91	600 ± log 0.323 log 0.458 ± 0.264 0.85	519 ± log 0.201 log 0.787 ± 0.293 1.0	713 ± log 0.143	1a : 1b < 2S.D. 1a : 2 < 2S.D. 1a : 3 < 2S.D. 1b : 2 > 2S.D. 1b : 3 > 2S.D. 2 : 3 < 2S.D.
4.	14. IX.	ED ₅₀ IS.D. α RP	b) 88.2 ± log 0.178 log 0.915 ± 0.306 5.89				

* ED₅₀ expressed as 10⁶ germs.

In accordance with the results of our preliminary experiments and also of those presented in *Table I*, vaccine Hu V 1 appeared to be remarkably superior to USV vaccines. Except for experiment N^o 2, the differences were highly significant. No significant differences were, however, found between the results of parallel examinations of the same vaccines, *i. e.* USV I and II (experiment 1, 3, 4) and Hu V 1 (experiment 4 a, b).

Results of comparative studies performed by using "corrected" Hu V 1 vaccine. As vaccine Hu V 1 appeared to be remarkably superior to the USV I, II vaccines, for further comparison the Hu V 1 vaccine had to be brought to the same level as USV I and II. Vaccine Hu V 1 was taken as being 6 times more effective than the USV preparations; thus apparently the 60 × 10⁹ germs contained in 1 ampoule (0.2 ml) of Hu V 1 were equal to 360 × 10⁹ NIH germs. If the content of an original ampoule was resuspended in 7.2 ml, the

suspension obtained contained 50×10^9 germs per ml as expressed in the sense of the NIH standard. Vaccine HuV1 was therefore used with this correction, in doses similar to those of the USV vaccine, *i. e.* 3000×10^6 , 600×10^6 and 120×10^6 germs per dose. Results obtained are given in Table II.

Table II
Comparison of corrected Hu V 1 to USV II and III

N° of experiment	Date		Hu V 1	USV II.	USV III.	LD ₅₀ ± S.D.	Significance
			1	2	3	4	5
1.	16. IX. 1955.	ED ₅₀ IS.D. a RP	428 ± log 0.143 log 1.245 ± 0.357 1.75	752 ± log 0.143 log 1.210 ± 0.350 1.0		2490 ± log 0.254	1 : 2 < 2S.D.
2.	7. III. 1956.	ED ₅₀ IS.D. a RP	1260 ± log 0.291 log 0.586 ± 0.278 1.61		2039 ± log 0.258 log 0.773 ± 0.314 1.0	1120 ± log 0.155	1 : 3 < 2S.D.
3.	8. V.	ED ₅₀ IS.D. a RP	1295 ± log 0.180 log 1.000 ± 0.328 0.62		801 ± log 0.203 log 0.787 ± 0.293 1.0	335 ± log 0.172	1 : 3 < 2S.D.
4.	6. VI.	ED ₅₀ IS.D. a RP	a) 315 ± log 0.241 log 0.701 ± 0.289 1.67		528 ± log 0.161 log 1.055 ± 0.325 1.0	798 ± log 0.323	1a:1b < 2S.D. 1a:3 < 2S.D. 1b:3 < 2S.D.
4.	6. VI.	ED ₅₀ IS.D. a RP	b) 269 ± log 0.247 log 0.715 ± 0.293 1.95				
5.	29. VI.	ED ₅₀ IS.D. a RP	583 ± log 0.179 log 0.916 ± 0.307 1.59		930 ± log 0.336 log 0.472 ± 0.268 1.0	1156 ± log 0.201	1 : 3 < 2S.D.
6.	3. VIII.	ED ₅₀ IS.D. a RP	803 ± log 0.203 log 0.786 ± 0.293 0.94		755 ± log 0.143 log 1.215 ± 0.349 1.0	607 ± log 0.101	1 : 3 < 2S.D.

* ED₅₀ expressed as 10^6 germs

According to the data presented in *Table II*, the described correction of the Hu V 1 vaccine was adequate. No significant difference could be demonstrated between the immunizing potencies of USV and UV 1 preparations. In experiment 4 the two parallel examinations of the Hu V 1 vaccine gave essentially similar results. All the data obtained justified the use of an appropriately corrected Hu V 1 vaccine preparation for further studies.

Examination of suspension N° 546/3. As by 1958 our supply of Hu V 1 vaccine was nearly exhausted, the preparation of a new standard appeared to be urgent. Details of the preparation of the stock suspension are given in "Materials and methods". In *Table III* the results of 8 successive examinations made within 3 and half a years with the stock suspension 546/3 are presented. This stock suspension served later for the preparation of Hu V 2 and 3 standards. In all examinations mice were immunized with 3000×10^6 , 600×10^6 and 120×10^6 germs per dose, respectively.

The protective value of suspension 546/3 appeared in every titration to be superior to the standard, but the differences were not significant. In agreement with the data of other authors the protective value of the merthiolate conserved suspension remained unchanged for 3 and a half years.

The higher ED_{50} value obtained in the last titration was evidently due to the commonly known variation in biological titrations; particularly as in the same experiment the ED_{50} value of the lyophilized standard vaccine was also low.

Comparison of Hu V 1, 2, 3 and the foreign standard vaccine preparations. In these experiments the 3 immunizing doses used were 3000×10^6 , 600×10^6 and 120×10^6 germs per dose, except for Hu V 2 and 3 in certain cases and for I V in general. In experiment N° 2 vaccine I V was also used in the above doses; while further only in considerably lower ones; in titration (a) of experiment 3, 600×10^6 , 120×10^6 and 24×10^6 ; in titration (b), 140×10^6 , 28×10^6 and $5,6 \times 10^6$ germs per dose were used. In experiment 4 and 7 doses similar to those used in titration (a) of experiment 3 were injected. In all these experiments vaccine Hu V 1 was taken as the standard and the protective values of all the other vaccines were referred to it. The results are presented in *Tables IV/a* and *IV/b*.

The results presented in *Tables IV/a* and *b* could be explained as follows. In none of the titrations could a significant difference be detected between BV and Hu V 1 vaccines. Vaccine preparations USV III and IV appeared to be somewhat weaker than BV and Hu V 1 in experiment N° 3; these differences, however, did not reach the limit of significance. Thus the results of our examinations suggested the above 3 vaccines to be of essentially similar potency. As mentioned previously, the USV standard preparations were generally used as prescribed, *i. e.* for 1 year after having been resuspended. USV III, however, which had been resuspended on February 4, 1956, and stored at 4° C, exhibited

Table III
Comparative test with the suspension 546/3

N° of experiment	Date	Hu V ⁺⁺		RP	546/3		RP	LD ₅₀ ± S.D.	Significance
		ED ₅₀ ± 1 S.D.	α		ED ₅₀ ± 1 S.D.	α			
1.	3. VIII. 1956	803 ± log 0.203	log 0.786 ± 0.293	1.0	343 ± log 0.284	log 0.572 ± 0.275	2.34	607 ± log 0.102	< 2 S.D.
2.	3. IV. 1957	765 ± log 0.159	log 1.055 ± 0.325	1.0	330 ± log 0.159	log 1.128 ± 0.342	2.32	425.7 ± log 0.257	< 2 S.D.
3.	1. X. 1957	450 ± log 0.129	log 1.430 ± 0.392	1.0	98 ± log 0.460	log 0.544 ± 0.296	4.56	331 ± log 0.131	< 2 S.D.
4.	15. I. 1958	478 ± log 0.143	log 1.215 ± 0.345	1.0	158 ± log 0.258	log 0.815 ± 0.325	3.02	1622 ± log 0.105	< 2 S.D.
5.	9. IV. 1958	620 ± log 0.271	log 0.577 ± 0.272	1.0	370 ± log 0.238 510 ± log 0.180	log 0.687 ± 0.286 log 0.945 ± 0.314	1.67 1.21	2940 ± log 0.256	< 2 S.D. < 2 S.D.
6.	30. IV. 1958	162 ± log 0.202	log 1.028 ± 0.364	1.0	155 ± log 0.146	log 1.500 ± 0.486	1.04	2300 ± log 0.210	< 2 S.D.
7.	2. VII. 1958	382 ± log 0.142	log 1.272 ± 0.365	1.0	101 ± log 0.183	log 1.459 ± 0.554	3.77	597 ± log 0.114	< 2 S.D.
8.	27. I. 1960	1179 ± log 0.138	log 1.358 ± 0.389	1.0	1050 ± log 0.141	log 1.300 ± 0.375	1.12	665 ± log 0.103	< 2 S.D.

* ED₅₀ expressed as 10⁶ germs.

Table IV/a
Comparison of vaccines Hu V 1, Hu V 2, and BV, I V and USV III, IV

N° of experiment	Date		Hu V 1	Hu V 2/1	Hu V 2/2	Hu V 2/3	BV	I V	USV III	USV IV	LD ₅₀ ± S.D.	Significance
			1	2	3	4	5	6	7	8	9	10
1.	1. X. 1957	ED ₅₀ * ±1 S.D. a RP	450 ± log 0.129 log 1.430 ± 0.392 1.0				518 ± log 0.200 log 0.787 ± 0.293 0.87				519 ± log 0.201	< 2 S.D.
2.	9. IV. 1958	ED ₅₀ * ±1 S.D. a RP	620 ± log 0.271 log 0.557 ± 0.272 1.0				600 ± log 0.106 log 1.932 ± 0.522 1.03	130 ± log 0.335 log 0.540 ± 0.309 4.76	728 ± log 0.271 log 0.536 ± 0.271 0.85	600 ± log 0.201 log 0.787 ± 0.293 1.03	2490 ± log 0.256	< 2 S.D.
3.	2. VII. 1958	ED ₅₀ * ±1 S.D. a RP ED ₅₀ * ±1 S.D. a RP	a) 473 ± log 0.159 log 1.058 ± 0.325 1.0 b) 382 ± log 0.142 log 1.272 ± 0.365 1.23	a) 245 ± log 0.131 log 1.402 ± 0.382 1.93 b) 400 ± log 0.203 log 0.786 ± 0.293 1.18	a) 336.5 ± log 0.091 log 2.582 ± 0.754 1.40 b) 320 ± log 0.143 log 1.243 ± 0.357 1.47	a) 167 ± log 0.159 log 1.342 ± 0.432 2.83 b) 372 ± log 0.159 log 1.10 ± 0.335 1.27	600 ± log 0.131 log 1.387 ± 0.634 0.78	a) 165.5 ± log 0.336 log 0.755 ± 0.332 2.85 b) 77.5 ± log 0.336 log 0.472 ± 0.268 6.1	1085 ± log 0.159 log 1.128 ± 0.343 0.43	1175 ± log 0.138 log 1.355 ± 0.389 0.40	597 ± log 0.114	1a=6b> 6a=7> 5=6b> 6a=8> 7=6b> 1b=7> 8=6b> 1b=8> 2a=5> 3b=7> 2a=7> 3b=8> 2a=8> 4b=7> 3a=7> 4b=8> 3a=8> 4a=5> 4a=7> 4a=8>
4.	30. VII. 1958	ED ₅₀ * ±1 S.D. a RP	910 ± log 0.115 log 1.730 ± 0.462 1.0					120 ± log 0.201 log 0.787 ± 0.293 7.58			1622 ± log 0.159	1:6 > 2 S.D.

* ED₅₀ expressed as 10⁶ germs

Table IV/b

Comparison of vaccines Hu V 1, 3 and I V and USV IV

N° of experiment	Date		Hu V 1	Hu V 3/1	Hu V 3/2	Hu V 3/3	USV IV	I V	LD ₅₀ ± S.D.	Significance
			1	2	3	4	5	6	7	8
5.	27. VIII. 1958	ED ₅₀ ± 1 S.D. a RP ED ₅₀ ± 1 S.D. a RP	1452 ± log 0.1788 log 1.043 ± 0.339 1.0	a) 414 ± log 0.159 log 1.072 ± 0.332 3.51 b) 575 ± log 0.131 log 1.418 ± 0.384 2.64	a) 514 ± log 0.202 log 0.787 ± 0.293 2.83 b) 495 ± log 0.0915 log 2.475 ± 0.729 2.94	a) 609 ± log 0.173 log 1.158 ± 0.354 2.39 b) 483.3 ± log 0.083 log 0.286 ± 0.845 3.01	a) 1215 ± log 0.157 log 1.155 ± 0.353 1.19		< 200	1 : 2a> 1 : 3b> 1 : 4b> 2 S.D. 5 : 2a> 5 : 3b> 5 : 4b> 1 : 5< 2 S.D.
6.	2. X. 1958	ED ₅₀ ± 1 S.D. a RP ED ₅₀ ± 1 S.D. a RP ED ₅₀ ± 1 S.D. a RP	a) 206 ± log 0.255 log 0.744 ± 0.303 1.0 b) 342 ± log 0.142 log 1.302 ± 0.374 0.603 c) 518 ± log 0.201 log 0.787 ± 0.293 0.398	a) 179 ± log 0.209 log 0.972 ± 0.342 1.15 b) 495 ± log 0.271 log 0.572 ± 0.271 0.416					1903 ± log 0.179	< 2 S. D.
7.	6. XI. 1958	ED ₅₀ ± 1 S.D. a RP ED ₅₀ ± 1 S.D. a RP ED ₅₀ ± 1 S.D. a RP	a) 291 ± log 0.175 log 1.158 ± 0.353 1.0 b) 259 ± log 0.115 log 1.819 ± 0.496 1.12 c) 204 ± log 0.255 log 0.744 ± 0.303 1.42	a) 264 ± log 0.154 log 1.215 ± 0.368 1.1 b) 168 ± log 0.408 log 0.502 ± 0.278 1.73 c) 183.5 ± log 0.129 log 1.645 ± 0.500 1.58			448 ± log 0.202 log 0.787 ± 0.292 0.65	28.2 ± log 0.256 log 0.859 ± 0.336 10.3	1622 ± log 0.238	1a : 6> 1b : 6> 1c : 6> 2a : 6> 2 S.D. 2b : 6> 2c : 6> 5 : 6>

in experiments N° 2 and 3 performed on April 9 and July 2, 1958, a still undamaged potency. In other words after 2 years of storage this preparation was not significantly inferior to USV IV, BV and Hu V 1 resuspended fresh on April 9, 1958. Vaccine preparation I V appeared to be remarkably superior to BV, USV and Hu V 1 in every experiment. The difference was significant in 3 out of 5 experiments. This result was in agreement with those of other authors.

Data on parallel examinations of different Hu V 1 vaccine preparations are given in *Table IV/b*. Vaccine Hu V 1 was examined in experiment N° 3 in two (a, b), in experiments 6 and 7 in three (a, b, c) parallels each. The results of parallel titrations were sufficiently uniform, *i. e.* the differences found did not reach the level of significance. The differences between the results of titrations of the same material at different points of time did not exceed the usual variations of biological titrations.

Vaccine preparation Hu V 2 was examined in experiment N° 3. Two parallel examinations were performed with each 3 different types of dosage of this vaccine (2/1, 2/2, 2/3; see "Materials and methods"). The number of germs per ml varied as follows: 1500×10^6 , 300×10^6 , 60×10^6 for vaccine 2/1; 2250×10^6 , 450×10^6 and 90×10^6 for vaccine 2/2; 3000×10^6 , 600×10^6 , 120×10^6 for vaccine 2/3. Six parallel examinations were carried out with each preparation using 3 different types of dosage per experiment. This method was chosen to obtain data on the potency of different doses of the same vaccine. The results obtained were remarkably uniform and neither the two parallels of the single titrations nor the 3 different types of dosage yielded results of significant difference. All results uniformly suggested the superiority of Hu V 2 to Hu V 1. This result was, however, expectable on the grounds of our earlier favourable experience with suspension N° 546/3.

Vaccine Hu V 3 was examined in experiments N° 5, 6 and 7. In experiment N° 5 six parallel titrations were performed for each 2 tests (a, b), using the following doses: 3000×10^6 , 600×10^6 , 120×10^6 for 3/1; 2250×10^6 , 450×10^6 and 90×10^6 for 3/2; and 1500×10^6 , 300×10^6 and 60×10^6 for 3/3. No significant differences were found in the 2 parallel titrations or in the individual experiments where different doses of the vaccine were used. In experiments N° 6 and 7 two (a, b) and three (a, b, c) parallel titrations of Hu V 3 vaccine were performed. The immunizing doses in these experiments were uniformly 3000×10^6 , 600×10^6 and 120×10^6 germs per dose. Results similar to those of some earlier experiments were obtained also here, *i. e.* no significant differences could be demonstrated. As expected, vaccine Hu V 3 (prepared from the highly effective suspension N° 546/3) was superior in every experiment to vaccine Hu V 1.

Discussion

The reliability and validity of the active mouse protection test was effectively reinforced by the fact that the recent field studies performed under the auspices of the Medical Research Council [2, 3] revealed an intimate and significant correlation between the epidemiological effectiveness and the protective value in mouse test of pertussis vaccines. By this statement the use of the mouse protection test obtained a firm practical support and our knowledge about the standardization of pertussis vaccines was remarkably increased by recent investigations [12—21, 5, 6]. Studies in this field yielded valuable informations concerning technical details, sources of error and reproducibility of the active mouse protection test. The great number of experimental data accumulated was very correctly and exactly evaluated by statistical methods. This resulted in the final settlement of the technology of production of vaccine standards and their introduction into general practice.

The Expert Committee on Biological Standardization of WHO suggested the preparation of a lyophilized international standard vaccine [7]. This work was mostly based on the results obtained by the Medical Research Council trial and since that time the international standard was compared in several laboratories to USV and BV preparations. These studies uniformly proved [21] that while the potency of BV and USV was similar, the international reference vaccine was 5 to 6 times more effective depending on the actual conditions of the experiment.

In the present paper we have reported about the preparation and the comparative examinations of our own lyophilized standard pertussis vaccines. We could state that our Hu V 1 preparation had a potency similar to that of USV and BV, while significantly lower than that of I V. The reproducibility of our titrations was proven by the fact that no significant differences could be demonstrated between the results of parallel titrations of Hu V 1 vaccine.

The successive examinations performed on stock suspension N° 546/3 (used for production of Hu V 2 and Hu V 3 standards) showed that this preparation had not measurably lost its immunogenic potency during 3 and a half years' storage. This observation was in agreement with those of KENDRICK *et al.* [22], UNGAR and BASIL [23] and PITTMAN [14] who also demonstrated the stability of appropriately prepared and stored pertussis vaccines.

The numerous examinations carried out with Hu V 2 and Hu V 3 vaccines yielded remarkably uniform results, *i. e.* no significant differences were observed in parallel titrations or in titrations with different immunizing doses.

In our examinations the LD₅₀ dose of the virulent challenge strain was 200 to 2900 germs. Similarly to IRWIN and STANDFAST [18] and KRAG ANDERSEN and WEIS BENTZON [24] we also failed to demonstrate a correlation between the LD₅₀ dose of the challenge strain and the ED₅₀ dose of the vaccine.

IRWIN and STANDFAST [18], UNGAR and BASIL [16] and COHEN and LEPPINK [20] proved by precise statistical evaluation of their titration experiments that a great number of mice and repeated titrations are required for exactly establishing the potency of the single vaccine preparations. This statement must be regarded as valid in cases when for instance very precise determinations are needed to compare the epidemiological value and mouse protecting potency of a certain vaccine. Nevertheless, for routine examination of commercial vaccines a smaller number of examinations may probably yield adequate results. The studies of both PITTMAN [13, 14] and our own suggested that 2 or 3 repeated examinations are usually sufficient for common routine vaccine testing. On the basis of the general experience — keeping the role of individual deviations of the titrations in mind — a vaccine should be regarded as acceptable according to the NIH prescriptions [10] if its total human dose contains 8 to 36 protective units as referred to a standard vaccine of 12 protective units. According to the opinion of ARMITAGE and PERRY [5], vaccines having a 1.2 to 6.4 times higher protective value than that of the BV should also be regarded as acceptable. The protective value of the lyophilized standard vaccine Hu V 3 which is used as the Hungarian standard, was 1.5 times higher than that of Hu V 1 vaccine, the latter being equivalent to the BV and USV standards. Thus we think that any vaccine having a protective value similar to that of the Hu V 3 standard should be accepted for commercial use. The precise standardization of the titration methods will greatly contribute to the reliability of the final results. We could add that possibly several (10 to 12) vaccines should simultaneously be titrated in one experiment, thus facilitating evaluation by the availability of a number of comparable data. Last but not least, standardization of the production method also appears to be important, it being most probable that similarly produced vaccines will have similar potencies. It is apparent, however, that we still lack an appropriate standard vaccine for the evaluation of the potency of adsorbed vaccines, as the dose-response-curves of the latter differ remarkably from those of plain vaccines. We hope that the studies in progress will soon bring a solution to this problem.

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Address of authors:

ISTVÁN JOÓ, ZSUZSANNA PUSZTAI, VERA P. JUHÁSZ

Institute for Serobacteriological Production and Research "Human", Szállás u. 5—7., Budapest X., Hungary.

DISTRIBUTION OF SHIGELLA FLEXNERI SEROTYPES IN HUNGARY BETWEEN 1945 AND 1958

By

ADELE VERTÉNYI

Institute of Microbiology, University Medical School, Pécs

(Received April 30, 1960)

Summary. (i) In the years from 1945 to 1958 the type of 2156 *Sh. flexneri* strains has been determined. Type 2a was the most common (65.8 per cent), the next in frequency was type 3 (18.6 per cent), the other types ranged between 0.1 to 2.8 per cent. Regarding the dynamics of type distribution, the growing predominance of type 2a was accompanied by an increase in the number of infections due to type 3, whereas types 1b and 6 exhibited tendency to occur less frequently.

(ii) Responsibility for epidemics may be practically ascribed to types 2a and 3.

(iii) The geographical distribution of *Sh. flexneri* types essentially corresponded to the average values, but in the southern and eastern regions of Hungary type 3 was more frequent. In some areas there was a relative local preponderance of some types [4a, 6].

(iv) In chronic carriers practically all types were found to occur. Variant Y deviated from the average values reflecting the proportional incidence of the different types, a fact pointing to the minus variant character of that variant.

(v) Our findings have been compared with the pertaining results obtained by other authors, and attention has been drawn to the importance of type identification.

Systematic identification of *Shigella* types has been rendered indispensable by epidemiological requirements and also by the attempts at specific prevention. Year after year, our institute strives to arrive at a true estimate of the situation and of the changes in type distribution. The strains constituting the material for the identification of types belonged chiefly to the *Sh. flexneri* group, therefore our studies have furnished data on the incidence of this group.

As to the proportional occurrence of various *Shigella* groups in Hungary after World War II, SERÉNY [1] found *Sh. flexneri* strains in 57 per cent, *Sh. sonnei* in 41 per cent, *Sh. dysenteriae* 1 in 0.4 per cent, and *Sh. dysenteriae* type 2 in 1 per cent. Among the vast number of strains received by us for identification, we found no strains belonging to *Sh. dysenteriae* 3 to 7. On the other hand, we identified several types of *Sh. boydii*, namely 2 strains of type 1, and 1 each of types 3, 5 and 6.

Accordingly, we wish to report only on our studies concerned with the incidence of *Sh. flexneri* types. The distribution of types was found to agree practically with the European pattern [2, 3]; we nevertheless think that the few deviations and the dynamics observed in the changes of type distribution may justify the publication of our findings.

Materials and methods

Strains. — Determinations were carried out only in part on strains isolated from the routine material of our institute; the greater part of the strains originated from various parts of the country and was made available at our request.

Determination of types. — All strains were tested for biochemical properties; in general, NÓGRÁDY's polytropic culture medium [4] was used. Slide agglutination was performed in polyvalent sera, as well as in factor sera. The latter were prepared according to RAUSS *et al.* [5, 10], with some modifications. Some of them were made at the "Human" Institute for Vaccine Production and Research after our prescription.

The types were designated according to the international nomenclature [6].

Results

In the years from 1945 to 1958, 2156 strains were identified.

Table I

Distribution of Shigella flexneri types in relation to time

Types	1945—1950		1951—1952		1953—1954		1955—1956		1957—1958		Total	
	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%
1a	3	1.8	3	0.7	1	0.2	2	0.3	1	0.1	9	0.4
1b	31	18.7	1	0.2	1	0.2	17	2.9	10	1.7	56	2.5
2a	74	44.6	292	66.7	301	71.4	416	71.1	337	59.2	1420	65.8
2b	5	3.0	30	6.8	4	0.9	11	1.8	1	0.1	51	2.4
3	15	9.0	71	16.2	74	17.5	64	10.9	194	34.0	400	18.6
4a	6	3.6	5	1.1	6	1.3	12	2.0	13	2.2	42	2.0
4b	3	1.8	1	0.2	0	0	0	0	0	0	4	0.1
5	3	1.8	0	0	0	0	0	0	1	0.1	4	0.1
6	21	12.7	12	2.8	8	1.9	13	2.2	5	0.8	59	2.8
X	3	1.8	11	2.3	15	3.5	21	3.5	2	0.3	52	2.4
Y	2	1.2	12	2.8	11	2.6	29	4.9	5	0.8	59	2.8
Total	166	100	438	100	421	100	585	100	569	100	2156	100

In Table I, the number and percentual distribution of identified *Sh. flexneri* types are presented in relation to age-groups and years. Data for the years from 1945 to 1950 are those of RAUSS, MAGYAR and KÉTYI [7]. Those for the years 1951 and 1952 have already been reported [8, 10].

Table I clearly demonstrates the dominant incidence of type 2a (65.8 per cent). The next in frequency was type 3 (18.6 per cent). The other types were less common, but all of them were encountered, in the following order of incidence. Variant Y and type 6 (2.8 per cent); type 1b (2.5 per cent); type 2b and variant X (2.4 per cent); type 4a (2.0 per cent). Types 5 and 4b were extremely rare (0.1 per cent). From 1952 type 4b did not occur in our material until

1958 when a group of monkeys suffering from dysentery excreted this type of organism.

Distribution according to age-groups and years naturally displayed fluctuations. First of all, type 2a had attained an increasing prevalence — culminating chiefly in the years 1955 and 1956 — until in 1957 and 1958 its incidence somewhat decreased, mainly in favour of type 3. Type 6 has practically lost its importance. The distribution of pathogenic types was definitely less than in the years 1945 to 1950 when the incidence of infection with types 2a and 3 amounted to 53.6 per cent. In 1957—1958 these organisms were

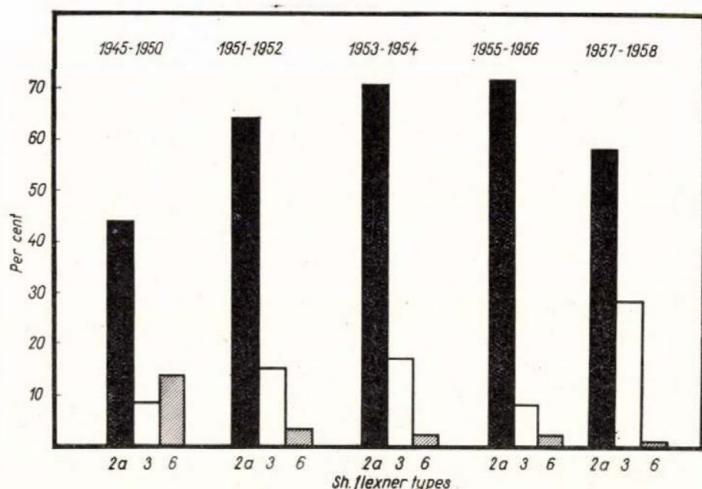


Fig. 1. Occurrence of *Sh. flexneri* types responsible for epidemics in the years from 1945 to 1958

responsible for 94.4 per cent of all *Sh. flexneri* infections. Other types were sporadic.

Although we did not perform epidemiological studies, from personal communications and the distribution of strains received at identical periods, types 2a and 3 alone seemed to have given rise to epidemics, while type 6 caused disease only in a few groups. The other types were responsible for sporadic cases only. In order to illustrate the distributional changes shown by these three types, in Fig. 1 the percentual incidence of types 2a, 3 and 6 is presented graphically for the years 1945 to 1950, as well as for periods of two years.

Fig. 1 clearly demonstrates some important data from Table I, namely the increasing incidence of type 2a until 1956, and of type 3 during 1957—1958, as well as the gradually diminishing frequency of type 6.

The great majority of our strains originated from Transdanubia, mainly the town Pécs, further Budapest and its environment. An attempt has never-

theless been made to give a geographic outline of type distribution which consequently presents a somewhat contorted picture in the eastern parts of Hungary.

Table II
Geographical distribution of *Shigella flexneri* types 1953—1958

Types	Southern Transdanubia		Central Transdanubia		Northern Transdanubia		Budapest		North-eastern Hungary		Great Plain		Total	
	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%
1a	0	0	0	0	0	0	2	0.3	0	0	0	0	2	0.1
1b	7	1.4	6	3.0	0	0	10	1.8	4	4.9	0	0	27	1.7
2a	303	61.4	135	68.1	98	79.0	386	71.8	63	77.7	45	41.6	1030	66.8
2b	2	0.4	2	1.0	0	0	7	1.3	0	0	0	0	11	0.7
3	162	32.8	34	17.1	11	8.8	71	13.2	11	13.5	54	50.0	343	22.2
4a	5	1.0	14	7.0	0	0	3	0.5	0	0	3	2.7	25	1.6
4b	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	1	0.2	0	0	0	0	0	0	0	0	0	0	1	0.06
6	4	0.8	2	1.0	11	8.8	9	1.6	0	0	1	0.9	27	1.7
X	5	1.0	3	1.5	3	2.4	13	2.4	2	2.4	0	0	26	1.6
Y	4	0.8	2	1.0	1	0.8	36	6.7	1	1.2	5	4.6	49	3.1
Total	493	100	198	100	124	100	537	100	81	100	108	100	1541	100

Table II contains the results of studies concerned with the country-wide incidence of *Sh. flexneri* types identified in the years 1953 to 1958. The general predominance of type 2a is manifest, closely followed by type 3. In the Great Plain the incidence of type 3 was higher (50 per cent) than that of type 2a (41.6 per cent); type 3 was common (32.8 per cent) also in southern Transdanubia. The other types were encountered sporadically, yet some of them assumed a relative prevalence in certain regions. Thus, type 4a showed an incidence of 7 per cent in Central Transdanubia in contrast to the country-wide average of 1.6 per cent (including Central Transdanubia). Type 6 occurred in 8.8 per cent in Northern Transdanubia when its country-wide average amounted to 1.7 per cent. The relative predominance of the Y variant in Budapest — 6.7 per cent — may be explained by the fact that these strains had originated mostly from chronic carriers. With due regard to these data it may nevertheless be stated that the distribution of *Sh. flexneri* types did not exhibit considerable differences in various parts of Hungary, and an adequately large material would have yielded strains of every type.

This report is not intended to deal with the question of variants and their incidence. We nevertheless wish to emphasize the above-mentioned fre-

quency of the variant Y in association with chronic dysentery. Our findings in the case of 100 carriers are presented in *Table III*. Thirty to 40 of these were examined at least once every month over a period of six months to one year.

Table III
*Distribution of Sh. flexneri serotypes
in material from carriers*

Type	Number of Strains
1a	—
1b	1
2a	63
2b	2
3	12
4a	1
4b	—
5	—
6	1
X var.	1
Y var.	19
Total	100

The conclusion to be drawn from *Table III* may be summarized as follows. Material derived from carriers showed the same type distribution as that from subjects with acute disease, except in respect of the Y variant. The incidence of variant Y was remarkably high, 19 per cent, in contrast to the country average of 2.8 per cent. Our data did not permit of conclusions concerning degradation, but the high incidence in itself points to the biological position of the Y variant.

Discussion

Since the end of World War II a considerable change has taken place in the spread of Shigella groups. As all over Europe, in Hungary *Sh. dysenteriae* has practically lost its importance. Despite the invariably predominant position of *Sh. flexneri*, *Sh. sonnei* strains have been observed to gain ground. Other Shigella groups (*Sh. dysenteriae* 2, *Sh. boydii*) still occupy an inferior rank.

Though reliable identification of *Sh. flexneri* types has been possible only since World War II, certain types have undoubtedly become more common.

As early as 1947, YOUNG [9] already stressed the spread of types 2a and 3 all over the world and increase in incidence of type 6 in the USA.

In Europe, SEELIGER [2] reported in 1953 on the approximately equal dominant position of types 2a and 3, followed directly by types 2b and 4a. Types 5 and 4b did not occur in his material.

SLOPEK and METZGER [3] in 1958 dealt with the spread of *Shigella* types in Poland on the basis of very extensive material. Of more than 11 000 *Sh. flexneri* strains, 77 per cent were of type 2a; types 1, 3 and 4 were left far behind, showing an incidence of 6 to 7 per cent; type 5 did not occur at all.

Our own findings concerning the distribution of types also demonstrated the predominance of type 2a (66 per cent), the next in significance being type 3 with an incidence of 18 per cent. Other types played a subordinate role. Types 4b and 5 quite rare (0.1 per cent).

When the yearly distribution of types is studied in relation to age-groups, the first remarkable fact is the narrowing scale of the pathogenic types. Whereas the 166 strains identified between 1945 and 1950 had included all types, with type 2a dominating and followed closely by types 1b and 6, in the year 1958 types 5 and 4b did not occur among the 366 strains tested; type 3 alone was found as second to the dominant type 2a, while types 1b and 6 occupied an insignificant position.

Fig. 1 illustrating the distribution of the three types responsible for epidemics in Hungary (2a, 3, 6) reveals that epidemics were due practically to types 2a and 3.

As expected, the geographical distribution in Hungary of *Sh. flexneri* types did not display any remarkable difference in the characteristic distribution of types. A higher incidence of type 3 in the southern and eastern parts of the country has nevertheless been noted and the local predominance of some serotypes has been observed.

Our present findings have undoubtedly confirmed the importance of changes in type distribution from the aspects of epidemiology and prevention and are another proof of the variability of type distribution. The appearance of new serotypes may lead to unexpected epidemics in areas of low morbidity due to natural immunization. Although specific preventive vaccination is aimed at the bestowal of polyvalent immunity, the quantitative composition of the vaccine should be established with due regard to ensuring massive immunity to the commonest types. Knowledge concerning the incidence of types is indispensable for the epidemiological evaluation of preventive vaccinations and it is unnecessary to emphasize that the study of immunity to dysentery and the epidemiology of the disease must also rest on this basis.

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Address of the author:

ADÉL VERTÉNYI

Institute of Microbiology, University Medical School, Rákóczi út 2, Pécs, Hungary.

ISOLATION OF NEW STAPHYLOCOCCUS PHAGES AND THEIR USE IN PHAGE TYPING

By

HEDDA MILCH

State Institute of Hygiene, Budapest

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Summary. In order to identify non-typable *Staphylococcus* strains, phages were isolated from lysogenic *Staphylococci*.

The lytic activity of the new phages was studied on 865 strains. By making use of the new phages 46 per cent of previously untypable strains could be identified.

A new simple method has been developed for the preparation of specific anti-phage sera and a serological grouping of the phages has also been made.

When tested for resistance to antibiotics, *Staphylococcus* strains identifiable by the phages isolated exhibited the highest resistance. The untypable strains and those belonging to phage group III also possessed a remarkable resistance to antibiotics.

A nosocomial *Staphylococcus* epidemic observed in a newborn ward has been studied. Of the strains isolated 60 per cent belonged to phage type 81/304 (internationally known as type 81) and 23.3 per cent to 756/950 (untypable with basic-phages).

Phage type 756/950 has been found to possess a remarkable spreading and pathogenic potency and a comparatively high antibiotic resistance.

BURNET and LUSH [1] in 1934 were the first to isolate *Staphylococcus* phages. The starting point for the development of the present method was FISK's cross-culture method [2] and the observation that most *Staphylococcus* strains exhibited lysogenicity when tested by this method. FISK's statement was further developed by ROUNTREE's [3] observation that certain strains carried several different phages simultaneously. In 1945 WILSON and ATKINSON [4] isolated and adapted further phages; 11 of these were isolated by the method of FISK, and 7 were obtained by adaptation to new hosts. These latter phages yielded the basis for the development of the presently used phage series.

Most coagulase-positive *Staphylococci* are identifiable with the above type phages. Part of the strains untypable at routine test dilutions (RTD) could be typed when tested by less diluted phages [5, 6]. A number of the strains, however, were still untypable with phages. This seemed to be of importance in certain centres the untypable *Staphylococci* formed an important part of the routinely isolated strains. Of the 1002 strains examined in our laboratory in the year 1959, 289 strains (28.8 per cent) were untypable. This figure was in good agreement with the international data.

In order to decrease the number of untypable strains an attempt was made to isolate new phages, thus making epidemiologically evaluable data

available also for this group of Staphylococci. In the present paper our studies concerning isolation and use of new phages will be presented. After isolation and propagation of the new phages they were examined as to their applicability for typing purposes. Their grouping was also attempted on the basis of their lytic activity and serological characteristics. The lytic spectrum of our phages was first determined on 22 test-strains. Later their lytic reactions were studied on 865 random Staphylococcus strains isolated from routine bacteriological samples. For serological characterization of our new phages their homologous immune sera were also prepared.

Materials and methods

Phage isolations were attempted from Staphylococcus strains obtained from patients and nurses of different children's hospitals.

Most of the Staphylococcus strains originated from patients with staphylococcal infections treated at the *Paul Heim Children's Hospital, Budapest*. The further strains examined were obtained from the surgical departments of different Budapest hospitals, from the *Obstetric Department of the Péterfy Hospital*, further from patients with staphylococcal infections at different country hospitals and from cases of food poisoning. The actual sources of the single strains were pyoderma, pemphigoid of infants, abscess, empyema, discharge from ear and nose, throat and trachea, faeces, haemocultures, food, air, and different objects.

As a great number of Staphylococcus strains exhibited spontaneous lysis when stored at room temperature, or $+4^{\circ}\text{C}$, they appeared to be suitable for isolating phages.

Phage isolations were attempted from 40 Staphylococcus strains according to the prescriptions of ROUNTREE [3] and WAHL and FOUACE [7]. The bacteria were inoculated into Witte-peptone broth and incubated for 4 to 5 hours at 37°C . The cultures were then kept at $+4^{\circ}\text{C}$ temperature overnight. This was followed by centrifugation at 3000 r. p. m. for 10 minutes and the filtration of the supernatant through G 5 glass filters.

By this method we succeeded in isolating phages from 23 (57.5 per cent) of the 40 strains examined. The phages thus obtained initiated different grades of lysis of standard type strains, while they did not lyse their original hosts.

Out of the 23 phages isolated 4 were selected and propagated, the others were either of very low titre and lost or they had the same lytic spectra as one or the other of the 4 strains selected.

History of the 4 selected phages: Phage 304 was isolated from a lysogenic Staphylococcus strain obtained from the ear discharge of a patient with otitis. Its phage pattern was 79/80 at routine test dilution (RTD) and 52/52A at 1000xRTD. The phage was propagated on the standard host strain designated as No. 80.

Phage 377 was isolated from a lysogenic Staphylococcus strain obtained from the nasal discharge of a healthy adult. It was untypable by type phages both at RTD and at 1000xRTD. It was propagated on standard host strain No. 7.

Phage 756 was isolated from a lysogenic Staphylococcus strain obtained from pus. Its phage pattern was not typable at RTD and 7/47/54 at 1000xRTD. It was propagated on standard host strain No. 6.

Phage 950 was isolated from a lysogenic Staphylococcus strain obtained from nasal discharge. When tested at RTD it was of phage-type 53, at 1000xRTD of 7/54/75/77. It was propagated on standard host strain No. 53.

In further typing of our Staphylococcus strains we used beside the basic ones also our phages. The appropriate RTD and lysis spectrum for the latter was determined previously. The basic phages used were

Group I: 29, 52, 52A, 79, 80

Group II: 3A, 3B, 3C, 55, 71

Group III: 6, 7, 42E, 47, 53, 54, 73, 75, 77

Group IV: 42D

Group miscellaneous 81, 187.

The antibiotic sensitivity of the *Staphylococci* isolated was also determined. This was made by making use of the antibiotic-soaked paper discs prepared by the "Human" Institute for Serobacteriological Production and Research, Budapest. The antibiotics used were penicillin, streptomycin, chlortetracycline, oxytetracycline, tetracycline, chloramphenicol, neomycin, polymyxin B, erythromycin.*

Serological identification of the new phages isolated. BURNET in 1933 [8] and CRAIGIE in 1940 demonstrated that by making use of appropriately prepared antiphage sera, the phages might be grouped on a serological basis. The first serological studies on *Staphylococcus* phages were made by BURNET and LUSH [1] in 1935. ROUNTREE in 1949 stated [10] that phage neutralizing antibodies can be obtained by immunizing rabbits with *Staphylococcus* type phages and the type phages might be divided in serologically different groups by means of such sera. Nine serological groups of *Staphylococcus* phages were described by RIPPON [11] in 1956. The groups were designated as A, B, F, C, D, G, H, J, K. The phages used at present for typing belong to 4 serological groups, namely to A, B, F and L.

Preparation of anti-phage immune sera was carried out by the intravenous injection of a series of doses increasing from 0.5 to 2.0 ml at intervals of 3 days. A total of 5 to 6 doses was usually administered. Titre values were checked by bleeding on the 7th day after the last injection; in case of unsatisfactory titres immunization was continued. Sometimes the process was quite slow. A shortening of the immunization period was attempted by application of the intraperitoneal method described by NICOLLE *et al.* [12]. It was found that 5 days after the intraperitoneal injection of 145 ml phage material antibodies of fairly high titre became demonstrable. The peak was reached on the 20th to 22nd day but some of the phages were toxic and killed the rabbits in one day. To achieve immunity in rabbits with less phage material we later used the incomplete FREUND adjuvant [13], a mixture of 85 per cent Bayot F paraffine oil and 15 per cent of the detergent Arlacel A. A single dose consisted of a mixture of 15 ml adjuvant and phage material each, and it was given subcutaneously. Blood samples taken at the 5th, 10th, 15th and 20th day after the injection revealed a low antibody titre at the 5th day which remarkably rose from the 10th day on, reaching the peak at the 20th day. We tried to immunize with as little as 2.0 ml of phage material mixed to an equal amount of Freund adjuvant; in this experiment rise of the antibody titre started on the 15th day after immunization and satisfactory levels were reached on the 20th day.

Phage neutralization tests were carried out according to the method of BURNET [8] as modified by ANDERSON and FELIX [14]. Tenfold dilutions of sera were mixed with equal amounts of phage dilutions. 0.2 ml of the phage dilution to be used ought to produce 50 to 200 plaques when spread over a plate culture of the appropriate strain. Phage and serum mixtures were incubated for 3 hours at 37° C and then overnight at +4° C before tested. The serum titres were expressed as the reciprocal of the highest dilution neutralizing round 80 per cent of plaques under the experimental conditions used. As controls mixtures of phage and broth, and of phage and normal serum were used.

Results

When determining the *lytic activity* of the new phages we found that phage 304 produced lysis in test strains belonging to different groups, while phages 756, 377 and 950 in strains of group III. Accordingly phage 304 was regarded as belonging to miscellaneous group and the others to group III. The lytic activity of the above strains was further tested on 865 bacterial strains isolated from routine samples. Results are demonstrated in *Tables I to IV*.

* The antibiotic sensitivity tests were made by Dr. P. BARANYAI at the laboratory of the Paul Heim Children's Hospital, Budapest.

Table I
Lytic reaction of routine strains with phage 304

Groups and some common types*	Number of strains examined	Strains lysed			
		No.		Per cent	
		RTD	1000 × RTD	RTD	1000 × RTD
I	117	21	5	17.9	4.2
II	30	1	1	3.3	3.3
III	63	19	18	30.1	28.5
Mixed**	114	5	3	4.3	2.6
80/81	64	52	3	81.2	4.6
Miscellaneous 81	64	37	1	57.8	1.5
187	43	0	0	—	—
Untypable at RTD	81	15	17	18.5	20.9
Untypable	289	14	3	4.8	1.0
Total	665	164	51	18.9	5.9

* No group IV strain was encountered in our routine material.

** Strains simultaneously belonging to several phage groups.

Table II
Lytic reaction of routine strains with phage 756

Groups and some common types*	Number of strains examined	Strains lysed			
		No.		Per cent	
		RTD	1000 × RTD	RTD	1000 × RTD
I	117	12	18	10.2	15.3
II	30	1	0	3.3	—
III	63	47	9	74.6	14.2
Mixed**	114	7	2	6.1	1.7
80/81	64	2	3	3.1	4.6
Miscellaneous 81	64	0	0	—	—
187	43	0	0	—	—
Untypable at RTD	81	25	26	30.8	32.0
Untypable	289	2	25	0.7	8.7
Total	865	96	83	11.1	9.6

* No group IV strain was encountered in our routine material.

** Strains simultaneously belonging to several phage groups.

Table III

*Lytic reaction of routine strains with phage 377**

Groups and some common types	Number of strains examined	Strains lysed			
		No.		Per cent	
		RTD	1000×RTD	RTD	1000×RTD
I	117	0	7	—	5.9
II	30	0	0	—	—
III	63	28	17	44.4	26.9
Mixed**	114	2	4	1.7	3.5
80/81	64	0	3	—	4.6
Miscellaneous 81	64	0	0	—	—
187	43	0	0	—	—
Untypable at RTD	81	5	7	6.1	8.6
Untypable	289	0	0	—	—
Total	865	35	38	4.0	4.4

* No group IV strain was encountered in our routine material.

** Strains simultaneously belonging to several phage groups.

Table IV

*Lytic reaction of routine strains with phage 950**

Groups and some common types	Number of strains examined	Strains lysed			
		No.		Per cent	
		RTD	1000×RTD	RTD	1000×RTD
I	117	46	36	39.3	30.7
II	30	4	2	13.3	6.6
III	63	60	3	95.2	4.8
Mixed**	114	21	5	18.4	4.3
80/81	64	2	4	3.1	6.2
Miscellaneous 81	64	0	0	—	—
187	43	0	0	—	—
Untypable at RTD	81	56	34	69.1	41.9
Untypable	289	61	28	21.1	9.7
Total	865	250	112	28.9	12.9

* No group IV strain was encountered in our routine material.

** Strains simultaneously belonging to several phage groups.

As shown in *Table I* the highest incidence, 81.2 per cent of positive lytic reactions with phage 304, occurred among Staphylococci sensitive to strains 80/81. Phage 304 could be regarded as belonging to group miscellaneous according to its lytic effect. Out of the strains untypable at RTD and 1000×

RTD of the type-phages, 4.8 per cent yielded positive lytic reactions at RTD, while 1.0 per cent at $1000 \times$ RTD of phage 304. Thus use of the latter made it possible to type 5.8 per cent of hitherto untypable strains.

In *Table II* the lytic reactions of phage 756 are given. This phage gave lysis with 74.6 per cent of group III, therefore it belonged to the group III. Out of the strains untypable at RTD and $1000 \times$ RTD of the type phages 0.7 per cent yielded positive lytic reactions at RTD, and 8.7 per cent at $1000 \times$ RTD of phage 756.

By the use of phage 756, 9.4 per cent of the hitherto untypable strains could be typed.

Phage 377 (*Table III*) gave lysis with 44.4 per cent of phage group III strains, thus it appeared to belong to the same group. No lytic reactions with untypable strains could be demonstrated.

Phage 950 produced positive lytic reactions in 95.2 per cent of *Staphylococcus* strains belonging to group III. The phage belonged to the same group. Strains not giving positive lytic reactions at RTD and $1000 \times$ RTD of the type phages were lysed in 21.1 per cent at RTD and in 9.7 per cent at $1000 \times$ RTD of phage 950. The use of phage 950 resulted in the typing of 30.8 per cent of the hitherto untypable strains.

By the use of new phages we succeeded in typing 46 per cent of earlier untypable strains, among them 26 per cent at RTD and 19.4 per cent at $1000 \times$ RTD of the appropriate phages.

Results of the serological tests. The highest homologous antibody titres obtained by immunizing rabbits with the different phage strains were as follows: for 304, 10^{-3} ; for 756, 10^{-4} ; for 377, 10^{-7} ; and for 950, 10^{-5} .

Results of neutralization tests performed with the recently isolated phages are given in *Table V* and *VI*.

Anti-phage sera prepared with phages 304 and 377 neutralized those belonging to group A in dilutions as high as 10^{-3} and 10^{-4} , while sera containing antibodies against phages 756 and 950 neutralized those belonging to group B. Cross-reactions were obtained only in the case of phage 29, which gave reactions with anti-phage serum 304 at a dilution of 10^{-2} , and with 377 at 10^{-3} . In his serological studies RIPPON [11] succeeded in demonstrating that phages were usually neutralized only by their homologous antibodies. Weak cross reactions were, however, observed between the serological groups A and B.

Anti-phage sera A neutralized the phages 304 and 377, while anti-phage sera B neutralized the phages 756 and 950.

As revealed by our serological observations, phages 304 and 377 belonged to group A, while 756 and 950 to group B.

Results of typings. Since April, 1959, the recently isolated phages have been used for typing of *Staphylococci* isolated in our laboratory and elsewhere.

Table V

Neutralization of type phages by anti-phage sera prepared by the new phages

Serological group	Phages	Neutralization titre of anti-phage sera			
		304	377	756	950
A	3A	1000	1000	—	—
	3B	1000	> 1000	—	—
	3C	10 000	10 000	—	—
	6	> 10 000	10 000	—	—
	7	1000	100 000	—	—
	42E	1000	100 000	—	—
	47	1000	10 000	—	—
	54	< 1000	10 000	—	—
	73	100	10 000	—	—
	75	1000	10 000	—	—
	81	10 000	1000	—	—
B	29	100	100	> 1000	10 000
	52	—	—	> 1000	1000
	52A	—	—	10 000	10 000
	79	< 10	—	10 000	> 1000
	80	—	—	10 000	1000
	55	—	—	10 000	10 000
	71	—	—	> 1000	1000
	53	—	—	< 1000	1000
F	77	—	—	—	—
	42D	—	—	—	—
L	187	—	—	—	—
Homologous phages	304	1000	1000	—	—
	377	1000	100 000	—	—
	756	—	—	10 000	10 000
	950	—	—	10 000	100 000

The incidence of different phage types among Staphylococci originating from different diseases caused by this organism, are given in *Table VII*. Out of the 657 strains isolated 37.6 per cent belonged to phage type I. The distribution of group I strains according to their origin was as follows. Pyoderma, 46.1 per cent; air and objects, 52.6 per cent; post mortem material, 50.8 per cent. These results were in good agreement with those obtained in our survey study carried out in the patients and nurses of the infant wards of the Paul

Table VI

Neutralization of new phages by anti-phage sera A, B, F and L

New phages	Neutralization titre of anti-phage sera			
	A	B	F	L
304	10^{-7}	10	—	—
377	10^{-9}	10^{-1}	—	10
756	—	10^{-5}	—	—
950	—	10^{-4}	—	—

Heim Hospital, at the end of 1958 [15]. Strains obtained from cases of food poisoning were exceptional, as belonging mostly (53.8 per cent) to phage group III.

Table VII

Incidence of different phage types of strains of Staphylococcus aureus according to the sources of isolation

Sources of isolation	No. of strains examined	I	II	III	Mixed	Misc.	80/81	304	756/950
Pus	241	46.1	4.5	5.8	7.1	—	27.4	2.9	6.2
Ear	41	39.0	2.4	19.6	7.3	2.4	12.2	—	17.1
Nose	107	36.4	10.3	11.2	7.5	4.7	15.0	0.9	14.0
Throat, trachea	71	21.0	9.8	27.0	4.2	—	15.5	4.2	18.3
Faeces	81	26.0	4.9	6.2	3.7	—	32.2	1.2	25.8
Navel of newborn, breast of mother	25	24.0	—	8.0	12.0	4.0	4.0	—	48.0
Air, objects	19	52.6	—	—	—	—	42.1	5.3	—
Food poisoning (Food or discharge)	13	—	15.5	53.8	30.7	—	—	—	—
Post mortem material	59	50.8	3.4	15.2	5.1	—	11.9	1.7	11.9
Total	657	37.6	5.7	12.4	6.6	1.1	21.0	2.1	13.5

It was remarkable that from different discharges of infants and their contacts Staphylococci identified with our phages as type 756/950 were isolated in 48 per cent.

As revealed by our more recent studies, the above strain caused an epidemic of pyoderma among the newborns of an obstetric ward. For a long period following the epidemic Staphylococci of phage type 756/950 could be isolated from the nasal and throat discharge, the navel and skin of the infants as well as from the air of the ward.

Examination of antibiotic sensitivity. The correlation between antibiogram and phage sensitivity of 558 *Staphylococcus* strains was examined. The results are presented in *Table VIII*.

Table VIII
Correlations between resistance to antibiotics and phage type

Phage type	No. of strains examined	No. of antibiotics ineffective
I	187	5.8
II	28	6.3
III	35	6.8
Mixed	25	6.3
80/81	27	6.1
80/81/304	47	6.4
756/950	49	8.4
304	10	8.7
Not typable at RTD	119	7.3
Not typable	31	7.2
Total	558	6.6

All strains appeared to be resistant to an average of 6.6 antibiotics out of those tested. A resistance higher than the average was exhibited by strains belonging to phage group III and by the untypable strains. The highest resistance was encountered among strains identifiable by the phages isolated by us. Strains of type 304 and those of type 756/950 were resistant to 8.8 and 8.4 % antibiotics, respectively.

On examination of the antibiograms of the strains isolated in our survey of the previous year, those belonging to phage group III exhibited the highest resistance [15].

After having isolated and examined our new phages, they were used in a study of a *Staphylococcus* epidemic in the town Miskolc [16]. The epidemic started in June and July, 1959, at the Infants' Department of the City Hospital, usually with furunculosis and other pyodermas, often complicated with otitis and enteritis.

Of the strains isolated during the epidemic 86 were phage typed. Results of typing experiments are presented diagrammatically in *Fig. 1*.

Out of the 86 strains 52 (60.5 per cent) belonged to type 81/304, 3 strains to phage type 81 or 80. As the latter had probably originated from type 81/304 strains, they were tentatively included in the column for strains of type 81/304. These changes in type were probably brought about by lysogenic agents or

spontaneous transformation. Among the other strains 20 (23.3 per cent) belonged to phage type 756/950 and 7 (18 per cent) to other types (29; 7/47/54/75/950; 6/7/47/54/75/756/950; 187 and mixed). Four strains were untypable. Of these one turned out to harbour a free phage, thus being unsuitable for phage typing. It could not be excluded, however, that this strain had previously belonged to some known type. Out of the 86 strains 62 (72 per cent) were typable with international standard phages. Further 20 strains (23.3 per cent) could be

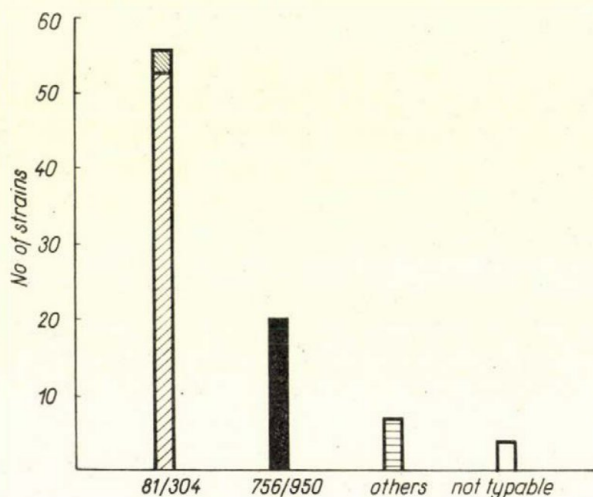


Fig. 1. Distribution of *Staphylococcus* strains from the Miskolc epidemic according to phage types

typed by our strains. Thus we succeeded in typing a total of 95.3 per cent of the isolated strains.

On the basis of our examinations it seemed very probable that 2 phage types of *Staphylococci* had predominated during the epidemic period. The strains isolated from the throat, nose, skin, pus and faeces of sick infants belonged to phage type 81/304 and 756/950. Out of the strains isolated from the hands of nurses, from the air of the wards, the napkins and the furniture, 7 belonged to phage type 81/304. In one case we succeeded in isolating from the hand of a nurse a type 756/950 strain.

Repeated examinations during the epidemic were made in 10 cases. In 7 of them the same type was isolated repeatedly. As revealed by the antibiotic sensitivity determinations the highest incidence of polyresistance was encountered among strains belonging to phage type 756/950. When comparing the clinical symptoms produced by *Staphylococci* belonging to group 81/304 and

756/950 the following observations were made. Among the patients infected with strains of phage type 81/304 pyoderma was observed in 62 per cent, fever in 9 per cent, loss in body weight in 6 per cent, enteritis in 38 per cent and otitis in 35 per cent. The incidence of symptoms among infants infected with type 756/950 strain was pyoderma, 42 per cent; fever 41 per cent; loss of body weight, 50 per cent; enteritis, 75 per cent; otitis, 50 per cent. Deaths amounted to

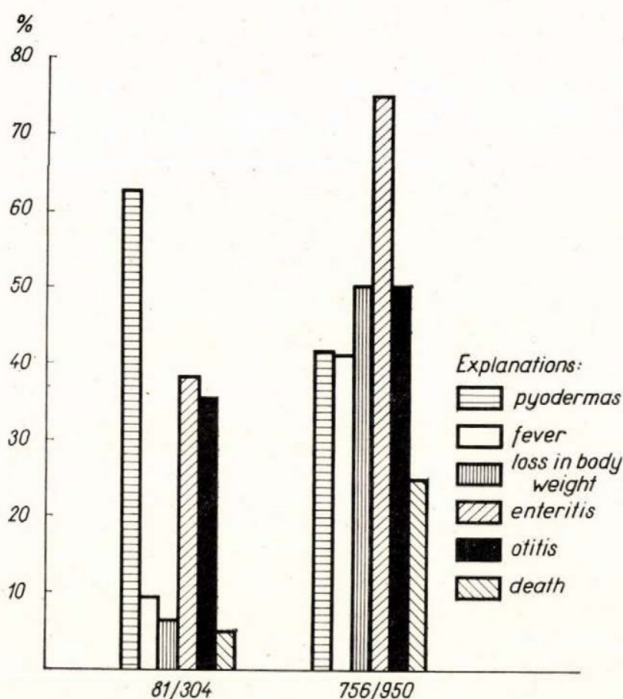


Fig. 2. Distribution of clinical symptoms and deaths according to the phage types of the agents

5 per cent and 25 per cent with strains 81/304 and 756/950, respectively. The data presented in Fig. 2 clearly demonstrate that although type 81/304 was more widely spread and caused pyoderma more often, the really severe symptoms (fever, loss in body weight, enteritis and otitis) were observed in infants infected with type 756/950 (See Fig. 2).

Discussion

Owing to the frequent incidence of different *Staphylococcus* strains the phages specific for *Staphylococci* are also often encountered. If the formation of the *Staphylococcus*-phage system results in lysogenization of the host,

certain changes in its phage sensitivity and other characteristics may occur. This seems to be the explanation for the development of new phage types in different geographical areas and for the development of untypable strains from those previously typable or vice versa.

This has made the authors in different countries to isolate phages from the local *Staphylococcus* strains and to use them after an appropriate adaptation for typing of previously untypable strains.

New *Staphylococcus* type phages may be obtained either by adaptation of a standard phage to a moderately susceptible host, or by adapting a newly isolated phage to a sensitive *Staphylococcus* strain [17]. In our present study the new phages were isolated from lysogenic *Staphylococcus* strains, while for the propagation of the new phages one of the standard host strains was used. The phages thus obtained were then tested as to their possible applicability for identification of previously untypable strains.

For examination of the serological characteristics of the phages isolated homologous anti-phage sera were prepared. Attempts were made to avoid the toxic effect of certain phage strains in rabbits and to simplify the methods of immunization. Both problems were solved by the use of the incomplete Freund adjuvant which made possible the preparation of highly active antibodies by one single injection and the avoiding of toxic effects. Up to now the stimulating effect of Freund adjuvant on the antibody formation has been described only in connection with bacteria, toxins, toxoids and viruses [13]. We may state that the adjuvant has been found useful also for the preparation of anti-phage immune sera. Further studies are required, however, before the method may be introduced generally.

It was the introduction of serological and phage typing that has made the aetiological and epidemiological follow-up of nosocomial *Staphylococcus* epidemics possible. In 1934 ANDERSON [18] described an epidemic of pemphigus neonatorum in Denmark and Norway caused by a *Staphylococcus* belonging to a certain serological type. In 1952 OEDING [19] observed puerperal mastitis caused by strains belonging to the same single serological type. Similar instances of puerperal mastitis caused by penicillin-resistant *Staphylococci* were described by COLBECK [20] and WEBB [21]. ISBISTER *et al.* [22] described a nosocomial epidemic in Sidney in 1934. This epidemic characterized by different pyoderms of infants was caused by type 80 *Staphylococci*; the strain was therefore originated as "epidemic" and since its isolation it has been described in different countries as a strain of particularly high virulence.

In 1956 BYNOE *et al.* [23] isolated a strain of high virulence in certain Canadian hospitals. They designated it as type 81. This observation justified the opinion of ROUNTREE [24] who at the time of isolation of type 80 *Staphylococci* warned against the simplification that this might be the only strain causing epidemic infections or furunculosis in infants.

Out of the 2 types of Staphylococci we have isolated in the Miskolc epidemic, type 81/304 exhibited vigorous spreading but caused only mild symptoms in contrast to type 756/950. Strains belonging to type 756/950 were not identifiable with the basic set of phages. BASS and FELTON [25] reported about the incidence of unidentifiable strains isolated during a nosocomial epidemic in Texas. In this particular epidemic out of the severe cases 50 per cent were caused by strains 80/81 and 13 by untypable strains. The possibility that the type 756/950 strains isolated by us may be similar to those described by BASS and FELTON may not be excluded.

Since the first isolation of type 756/950 from a nosocomial epidemic at Miskolc, the same strain was repeatedly isolated in connection with some smaller epidemics. This type was found to have caused scarlatoid in a certain hospital, grave pneumonia of newborn babies at a day nursery in Budapest and severe pyodermas observed among newborn babies at an obstetric department of a Budapest hospital. Accordingly it seemed to be justified to designate this particular strain as "Hungarian Nosocomial Epidemic Strain".

In connection with the Miskolc epidemic two main types of strains were isolated from patients exhibiting Staphylococcal infections. None of the other types simultaneously present caused any clinical symptoms. The spreading potency of type 81/304 and 756/950 strains under the same hygienic conditions in the same community was found to be remarkably higher than that of the others simultaneously present. Out of the 2 main types isolated, type 81/304 was characterized by a higher spreading potency, while type 756/950 by greater pathogenicity.

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Address of the author:

HEDDA MILCH

State Institute of Hygiene, Gyáli út 2/6, Budapest IX., Hungary.

THE 1959 POLIOMYELITIS EPIDEMIC IN HUNGARY

By

O. RUDNAI

State Institute of Hygiene, Budapest

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Summary. (i) Hungary experienced a poliomyelitis epidemic with 1830 cases in the year 1959. The attack rate, 18.3 per 100 000 population, was higher than in any of the previous years, except 1957. The epidemic was caused by type 1 poliovirus.

(ii) The epidemic starting from Budapest had spread in the whole country. Incidence rate was the highest in Pest county (30.9 per 100 000), and in only four of the nineteen counties had remained under 10 per 100 000.

(iii) The epidemic curve began to rise in June and reached its peak in August (the Budapest curve already in July). It fell off more sharply than usually, probably as a result of the vaccination campaign conducted in face of the epidemic.

(iv) In 1959 the age specific attack rate was the highest in infants, in contrast to the previous years when the peak of the age curve was at one year. The relative participation of one- and two-year-old children was markedly lower in 1959 than during the period 1952—1957. The shift can be explained by the immunization with the Salk vaccine carried out systematically since 1957.

(v) The case-fatality-rate was low, 2.9 per cent of the total, 1.8 per cent among the vaccinated and 4.6 percent among the unvaccinated patients. It has been demonstrated that vaccination was an important factor at lowering fatality.

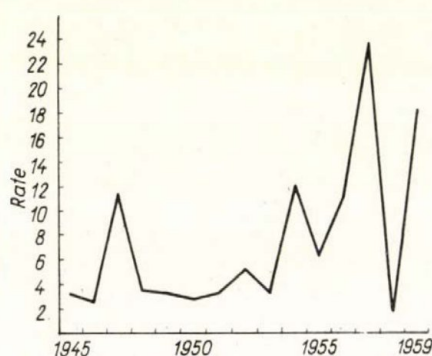
The incidence of poliomyelitis has shown a marked increase during the last decade. The average rate of 4.2 per 100 000 population for the years 1931—1938 rose to 8.5 (average for the period 1950—1957). The increase was continuous, *i. e.* the incidence was relatively high in the last few non-epidemic years, too. Up to 1945 the annual case incidence had never exceeded 1000 but in one year, since then it has exceeded 1000 in five years.

Characterization of the 1959 poliomyelitis epidemic. During the period 1945—1957 the attack rate 10 per 100 000 population was exceeded in 4 years, namely in 1947, 1954, 1956 and 1957 [1—4]. The 1957 epidemic was the greatest poliomyelitis epidemic in Hungary, with a case incidence of 2334 (nearly 24 per 100 000 population). In 1958 the number of reported cases was less than ever during the last 20 years, but a new epidemic broke out in 1959. The latter exceeded every poliomyelitis epidemic observed in Hungary before 1957. The number of notified cases was 1830, *i. e.* 18.3 per 100 000 population (*Table I and Fig. 1*).

Table I

Notified cases of, and deaths from, poliomyelitis in Hungary 1945—1959

Year	Cases		Deaths		Case-fatality-rate per cent
	number	per 100 000 population	number	per 100 000 population	
1945	280	3.1	32	0.4	11.4
1946	227	2.5	22	0.2	9.7
1947	1035	11.4	84	0.9	8.1
1948	314	3.4	22	0.2	7.0
1949	298	3.2	26	0.3	8.7
1950	257	2.8	22	0.2	8.6
1951	310	3.3	19	0.2	6.1
1952	500	5.3	29	0.3	5.8
1953	313	3.3	11	0.1	3.5
1954	1176	12.1	64	0.7	5.4
1955	617	6.3	18	0.2	2.9
1956	1100	11.2	77	0.8	7.0
1957	2334	23.8	143	1.5	6.1
1958	165	1.7	8	0.1	4.8
1959	1830	18.3	53	0.5	2.9

*Fig. 1. Poliomyelitis incidence in Hungary (attack rates per 100 000 population)*

The geographical distribution of the 1959 cases is presented in *Table II* and *Fig. 2*. *Fig. 3* shows the same for the previous three epidemic years.

The incidence rate for 1959 exceeded 10 per 100 000 in every county, except four. The rate was highest in Pest county, the one surrounding Budapest (30.9 per 100 000), and exceeded 20 per 100 000 in 5 more counties and in one town. The Budapest rate was higher than ever since the end of World

Table II

Poliomyelitis attack rates per 100 000 population for the 19 counties and 5 towns of Hungary, 1954—1959

County, town	1954	1955	1956	1957	1958	1959
<i>Counties</i>						
Baranya	7.5	7.0	3.1	8.7	1.0	18.5
Bács-Kiskun	7.5	6.8	10.5	23.3	1.4	19.8
Békés	8.4	2.3	3.6	24.0	1.1	16.7
Borsod-Abaúj-Zemplén	5.6	7.5	4.5	67.9	0.9	9.4
Csongrád	5.9	2.8	10.9	13.0	1.8	18.8
Fejér	16.1	4.9	9.7	13.4	2.8	25.3
Győr-Sopron	27.8	4.5	6.3	13.6	2.8	24.5
Hajdú-Bihar	13.8	5.9	3.2	88.2	1.8	9.9
Heves	10.5	4.3	3.1	40.6	0.9	19.5
Komárom	16.7	3.6	10.8	7.2	0.8	12.6
Nógrád	3.8	1.7	6.5	5.7	—	13.6
Pest	12.2	6.2	9.8	17.9	3.6	30.9
Somogy	8.8	6.1	5.6	11.9	0.3	8.6
Szabolcs-Szatmár	19.5	6.1	47.9	36.3	2.1	23.5
Szolnok	10.4	7.1	7.3	20.7	2.4	22.2
Tolna	8.3	2.9	3.3	11.5	—	7.8
Vas	2.8	2.1	8.0	10.0	0.7	13.4
Veszprém	44.4	7.9	8.4	12.4	3.2	23.9
Zala	3.3	8.5	2.2	15.6	1.1	14.2
Total for the counties	12.5	5.4	9.8	25.6	1.7	18.6
<i>Towns</i>						
Debrecen	6.2	6.2	10.8	16.2	0.8	13.2
Miskolc	5.3	10.0	8.7	69.3	0.6	11.8
Pécs	7.3	10.0	2.7	20.0	0.9	13.0
Szeged	15.0	5.0	8.0	23.0	—	25.0
Total for the country	12.3	5.6	9.7	26.2	1.6	18.4
Budapest	11.5	9.2	17.5	13.6	1.8	17.9
Grand total	12.1	6.3	11.2	23.8	1.7	18.3

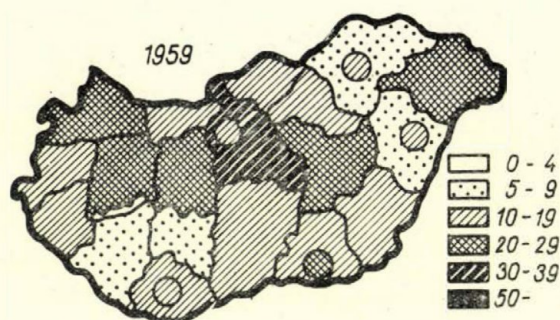


Fig. 2. Geographic distribution of poliomyelitis in Hungary (attack rates per 100 000 population)

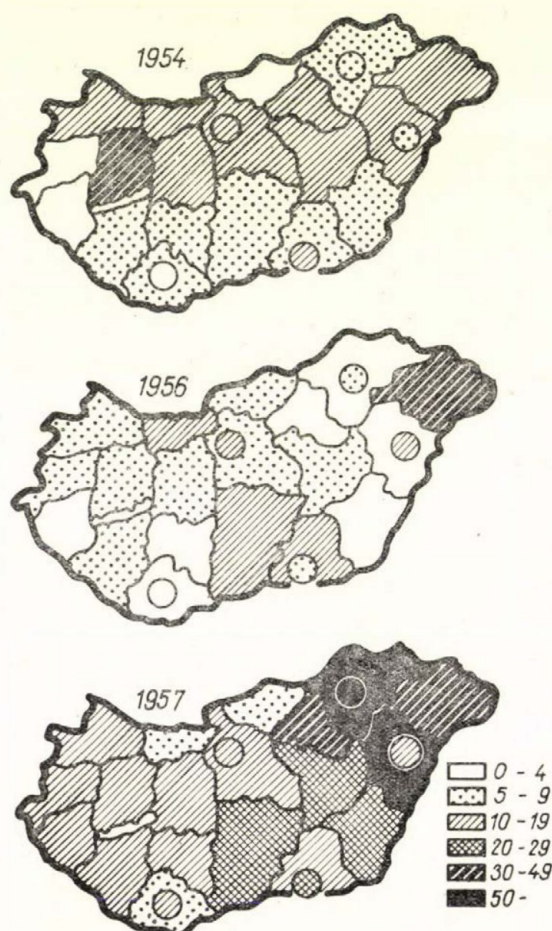


Fig. 3. Geographic distribution of poliomyelitis in Hungary (attack rates per 100 000 population)

War II. However, the high rates registered in 4 eastern regions in 1957 were not approximated in any of the counties and towns.

Fig. 4 shows the monthly incidence for the years 1957, 1958 and 1959.

In 1957 an increase in the number of cases was first observed in May, that was followed by a steep rise in June. The epidemic reached its peak in July and showed a sharp decline thereafter. The 1958 curve showed no seasonal fluctuation. It was unusual that more cases occurred during the first half of the year than in the second one. The lack of the seasonal peak can be ascribed to the interfering effect of the Bornholm epidemic wide-spread in Hungary during the second half of the year 1958 [5]. The Bornholm epidemic was caused by the Cocksackie B-3 virus.

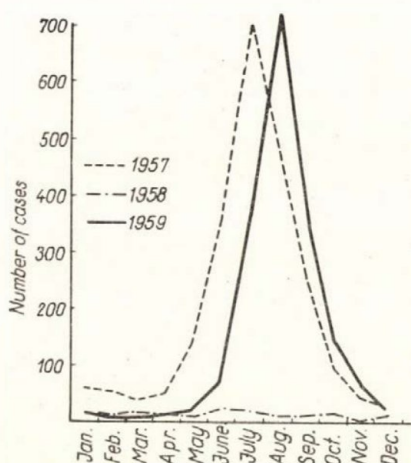


Fig. 4. Poliomyelitis incidence in Hungary, by months, in the years 1957, 1958 and 1959

During the first 4 months of 1959 the number of reported poliomyelitis cases remained even under the corresponding figures for the year 1958 and there was hardly any increase in incidence in May. In June, however, the epidemic became remarkable, and in July the number of reported cases was higher than ever before in that month, except 1957. The peak in August showed the highest monthly incidence of poliomyelitis ever registered in Hungary. The epidemic dropped in September even more suddenly than the 1957 epidemic did.

Fig. 5 shows the Budapest curve and the country curve for 1959.

The rise of the epidemic in Budapest preceded that in the country by a month. The Budapest epidemic reached its peak in July, but hardly less cases occurred in August. The country curve ran below the Budapest one up to July, but from August on, when the peak occurred, the rates for the

country were higher. The decline of the Budapest curve was sharper than that of the country curve.

Table III shows the percentual age distribution of the poliomyelitis cases in Budapest and of those in the country in the years from 1954 to 1959. We chose the percentage to characterize the age distribution, for no precise data on the geographical distribution of the population were available.

The 1959 data for the country showed an increase in the participation in the incidence of infants and of 3—5-year-old children, on the account of the one- and two-year-old children, but no change for the older groups. The Buda-

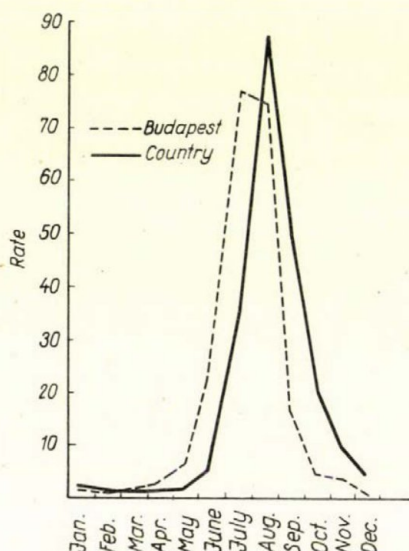


Fig. 5. Poliomyelitis seasonal incidence rates for Budapest and for the country, 1959 (per 100 000 population, on annual base)

pest data indicated a similar shift within the pre-school age groups, and a marked rise in the percentage distribution for the group over 15 years.

As seen in Table IV the age curve for the whole country peaked at one year of age in each year from 1952 to 1958. In three of these seven years the age specific attack rate for two-year-old children exceeded the rate for infants whereas in four years, including 1958, the latter rate was higher and in 1958 even approached the peak.

In contrast to this in 1959 the attack rate was the highest at 0 year of age and the age curve declined without any secondary peak.

In order to visualize the shift in the age distribution, for each calendar year the age specific attack rates were summed and the rates were expressed in per cent of the corresponding sum. The figures are presented in Table V.

Table III

Age distribution of poliomyelitis in per cent of the annual incidence, Hungary, 1954—1959

Age group in years	1954	1955	1956	1957	1958	1959
<i>a) Country</i>						
0	20.1	20.5	16.8	14.9	23.6	22.7
1—2	46.4	49.4	51.2	48.1	36.6	30.9
3—5	19.1	15.7	16.5	19.6	18.3	28.7
6—9	6.0	5.0	7.0	7.7	8.5	8.1
10—14	3.2	3.5	3.3	4.4	6.1	3.2
15+	5.2	5.9	5.2	5.3	6.9	6.3
unknown	—	—	—	—	—	0.1
Total	100.0	100.0	100.0	100.0	100.0	100.0
<i>b) Budapest</i>						
0	18.1	16.2	15.6	8.7	8.8	12.6
1—2	51.7	50.2	50.1	39.3	50.2	28.7
3—5	12.3	15.6	19.3	22.5	23.5	29.9
6—9	7.6	4.6	4.8	11.2	11.7	9.6
10—14	4.2	4.8	3.0	9.6	2.9	5.6
15+	6.1	8.6	7.2	8.7	2.9	13.6
unknown	—	—	—	—	—	—
Total	100.0	100.0	100.0	100.0	100.0	100.0
<i>c) Hungary</i>						
0	19.7	19.3	16.5	14.2	20.6	20.9
1—2	47.3	49.7	50.9	47.2	39.4	30.5
3—5	17.8	15.7	17.3	19.9	19.4	28.9
6—9	6.4	4.8	6.4	8.1	9.0	8.4
10—14	3.5	3.6	3.0	5.0	5.5	3.6
15+	5.3	6.9	5.9	5.6	6.1	7.6
unknown	—	—	—	—	—	0.1
Total	100.0	100.0	100.0	100.0	100.0	100.0

The data show that the age distribution was approximately constant between 1952 and 1957, and this uniformity lays particular stress on the shift which occurred in the years 1958 and 1959.

A detailed analysis of the incidence among infants is presented in *Table VI*.

Table IV

Poliomyelitis age specific attack rates per 100 000 population, Hungary, 1952—1959

Age in years	1952	1953	1954	1955	1956	1957	1958	1959
0	39.6	30.7	113.7	57.7	94.5	194.9	21.9	253.2
1	58.4	46.7	199.5	100.5	168.1	359.1	23.8	187.3
2	45.2	26.2	111.8	57.3	109.7	211.6	13.4	147.8
3	26.8	16.6	56.5	29.6	56.6	122.6	7.5	124.1
4	14.3	9.3	38.5	14.9	29.1	72.0	5.1	89.2
5	14.2	6.6	25.6	11.5	22.4	55.1	3.9	54.5
6—9	9.5	4.4	12.0	4.7	10.5	27.6	2.2	22.5
10—14	5.7	2.0	5.4	3.0	4.7	15.7	1.2	8.6
15+	0.8	0.4	0.8	0.6	0.9	1.7	0.1	1.9
Total	5.3	3.3	12.1	6.3	11.2	23.8	1.7	18.3

Table V

Age distribution of poliomyelitis in per cent of the summed age specific attack rates, Hungary, 1952—1959

Year	Number of cases	Per cent of the summed attack rates for the age groups						Total
		0	1—2	3—5	6—9	10—14	15 +	
		years						
1952	500	31.4	41.2	14.8	7.5	4.5	0.6	100.0
1953	313	36.2	42.9	12.8	5.2	2.4	0.5	100.0
1954	1176	34.6	47.7	12.2	3.6	1.6	0.3	100.0
1955	617	35.0	48.6	11.3	2.9	1.8	0.4	100.0
1956	1100	33.0	48.7	12.7	3.7	1.6	0.3	100.0
1957	2334	32.1	46.5	14.0	4.5	2.6	0.3	100.0
1958	165	44.5	37.2	11.2	4.5	2.4	0.2	100.0
1959	1830	46.7	30.7	16.5	4.2	1.6	0.3	100.0

The ratio incidence over half year of age per incidence under half year of age showed no change in 1959. However, the ratio incidence in the fourth quarter year of life per incidence in the third quarter year of life had decreased.

Regardless of slight fluctuations, the case-fatality-rates of poliomyelitis has been continuously declining since 1945 (*Table VI*, *Fig. 6*).

Table VI

Age distribution of the infant cases of poliomyelitis in Hungary, 1951—1959

Age in months	1951—57		1958		1959	
	Incidence					
	number	per cent	number	per cent	number	per cent
0—2	26	2.6	1	2.9	3	0.8
3—5	118	11.6	4	11.8	47	12.3
Total	144	14.2	5	14.7	50	13.1
6—8	339	33.4	10	29.4	157	41.0
9—11	533	52.4	19	55.9	176	45.9
Total	872	85.8	29	85.3	333	86.9
Grand total	1016	100.0	34	100.0	383	100.0

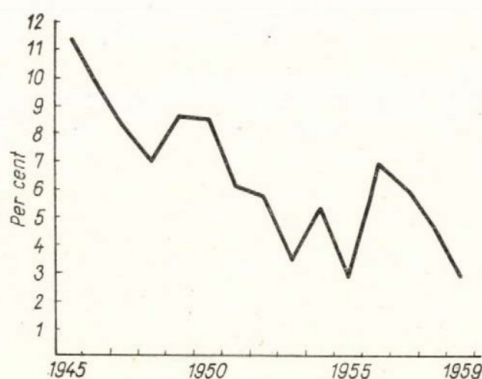


Fig. 6. Poliomyelitis case-fatality, Hungary, 1945—1959

In 1959 the case-fatality-rate was 2.9 per cent. Such a low rate had not been observed before, but in 1955.

The age distribution of fatality is presented in *Table VII*.

For the sake of comparison the average rates for the period 1949—1957 are also presented. The latter exceeded the 1959 rates in each age group. As during the previous years, in 1959, too, patients over 15 years of age showed the highest fatality; this age group was followed in order by the infants, the 2-year-old and one-year-old children. The age specific case-fatality-rate was approximately constant between 3 and 14 years of age.

Table VII
Poliomyelitis case-fatality-rates, by age, Hungary, 1949—1957 and 1959

Age in years	1949—57			1959		
	Number of cases	of these died		Number of cases	of these died	
		number	per cent		number	per cent
0	1064	81	7.6	383	16	4.3
1	1947	101	5.2	293	6	2.0
2	1224	51	4.2	265	9	3.4
3—5	1309	74	5.7	529	6	1.1
6—9	554	33	6.0	154	2	1.3
10—14	327	16	4.9	66	1	1.5
15	475	53	11.2	139	12	8.6
Unknown	5	—	—	1	1	—
Total	6905	409	5.9	1830	53	2.9

Discussion

In Hungary poliomyelitis showed a nearly regular cyclic fluctuation prior to World War II; epidemics followed each other at intervals of 5 or 6 years. This situation has considerably changed since 1952. During the six-years period 1954—1959 four years could be regarded as epidemic years. The disappearance of the cyclic fluctuation might be explained by two circumstances. (i) During epidemics only a part of the population, that living in circumscribed areas of epidemics foci, acquires immunity; *e. g.* in Szabolcs-Szatmár county (Eastern Hungary) poliomyelitis outbreaks were observed in three years during the four-year period 1954—1957, but none of the outbreaks extended to the whole county. Thus, in some districts of this county which represents about a twentieth of Hungary, the population which had remained unprotected was large enough to allow new epidemics to develop within short periods of time. (ii) The epidemics in the period 1954—1959 were caused by different types of poliovirus. According to the virological examinations carried on in Hungary since 1954, in 1954 type 2, in 1955 type 3 poliovirus was predominant [6, 7, 8], whereas the 1957 and 1959 epidemics were caused by type 1 virus [9, 10]. These two factors and the high pathogenicity of type 1 virus might explain the nation-wide spread of two epidemics within a three-year period.

In spite of the above evidence the 1959 epidemic was, to some extent, unexpected. It was hoped that the Salk vaccination carried out since 1957 would protect the otherwise most endangered age groups which had been vaccinated most systematically. The 1957 vaccination campaign has already

been evaluated [11, 12]; the effect of Salk vaccination on the 1959 epidemic will be evaluated in another report [13].

The geographical distribution of poliomyelitis in 1959, as well as in 1957, was characterized by attack rates exceeding 10 per 100 000 population in almost every county and town. This is in accordance with the fact that both epidemics were caused by the type 1 virus. The earlier epidemics affected smaller areas (Fig. 2), *e. g.* the 1947 epidemic the counties between the rivers Danube and Tisza, the 1954 epidemic extended to two counties in Transdanubia and to the northern part of Hungary. The 1956 epidemic afflicted the north-eastern corner of the country most seriously, and the attack rate exceeded 10 per 100 000 population in 3 other counties as well. In 1957 four north-eastern counties and the town Miskolc suffered most.

The 1959 epidemic starting from Budapest spread first to the surrounding Pest county, then all over the country. The attack rate was the highest in Pest county, the lowest in Tolna and Somogy counties (Southwest Hungary). The latter were free of epidemic during the previous five years. The attack rate remained under the level 10 per 100 000 population in two (Borsod-Abaúj-Zemplén and Hajdú-Bihar) of the four counties most seriously affected in 1957. The focus-forming property of poliomyelitis was apparent in 1959, too. There were circumscribed areas in both the epidemic and non-epidemic counties, where the attack rate highly exceeded the county's average rate.

In Hungary poliomyelitis is characterized by a well-defined seasonal fluctuation, the highest incidence being usually in August. This was the case in 1959, but the decline of the epidemic markedly differed from the usual decline. There were only 15 weeks when the incidence exceeded the weekly average of the same year in contrast to the usual 16—19 weeks. The drop of the epidemic (especially of the Budapest curve) was more sudden than of either of the preceding epidemics.

The rapid drop of the epidemic is thought to be due to the Salk vaccination performed during the epidemic. This view is supported by the fact that in Budapest, where the vaccination campaign had begun earlier, the epidemic curve fell off more sharply than in the country.

In Hungary the youngest age groups have suffered most from poliomyelitis, at least since the beginning of registration. Nevertheless, the participation of the youngest age groups has become more definite since World War II. The ratios of the age specific attack rates were approximately constant between 1952 and 1957, the rate for one-year-old children being the highest [14]. This situation had changed in 1958 and in 1959. In 1959, instead of the one-year-old children, the infants were showing the highest attack rate, and there was a marked decrease in the attack rate among the one- and the two-year-old children. It would be false to attribute this change in the age distribution to chance only. It chiefly resulted from the Salk vaccination, which had been

almost complete in the age group of 1 to 2 years while the majority of the infants had not been vaccinated, since most of these were vaccinated as late as during the 4th quarter of the first year of their life. Thus, a measurable effect of the vaccinations was the relative low incidence during late infancy. According to our data the participation of nine-to-eleven-months-old infants in the infant morbidity which had always exceeded 50 per cent, fell to 46 per cent in 1959.

The case-fatality-rate was 2.9 per cent. A low rate like this had occurred in one year only (1955). In that year, however, the less pathogenic type 3 poliovirus was being spread most widely. Thus, the fatality for 1959 may be considered as very favourable. The most important factors lowering it were the improvement in sanitary care as well as the rapid development as regards therapy. In 1959 all the patients, except four, were hospitalized. This is noteworthy, since in the past hospitalization reduced fatality by more than half. Since, however, during the previous few years hospitalization and therapy were almost as good as in 1959 and, in spite of this, fatality was definitely reduced in 1959, another factor must have been at play, and that was the vaccination. According to our analysis the case-fatality-rate for the patients immunized with the Salk vaccine amounted to 1.8 per cent in contrast to the 4.6 per cent rate for the unvaccinated patients.

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Address of the author:

OTTÓ RUDNAI

State Institute of Hygiene, Gyáli út 2-6, Budapest IX, Hungary.

АСТА MICROBIOLOGICA

ТОМ VII
РЕЗЮМЕ

МИКРОБИОЛОГИЧЕСКОЕ ОПРЕДЕЛЕНИЕ НЕБОЛЬШОГО КОЛИЧЕСТВА ХЛОРИДА

И. КОЛЛАР и М. ЯРАИ

Авторами был разработан метод для микробиологического определения хлорида на основе того принципа, что отдельные штаммы *Streptomyces aureofaciens* способны количественно использовать в определенных пределах концентрации, содержание хлорида находящейся в их распоряжении среды, для синтеза хлортетрациклина. Определением полученного хлортетрациклина исчислима первоначальная концентрация хлорида.

БИОХИМИЧЕСКИЕ ИССЛЕДОВАНИЯ С *STREPTOMYCES AUREOFACIENS* I. ИССЛЕДОВАНИЕ МЕХАНИЗМА ХЛОРИРОВАНИЯ

И. КОЛЛАР и М. ЯРАИ

1. Исследовалось действие ионов хлорида при биосинтезе хлортетрациклина; были получены данные согласно которым встраивание атома хлора в молекулу тетрациклина происходит в ранней фазе синтеза.

2. Результаты исследований, проведенных галоидными гомологами подкрепили прежние установления относительно тормозящего действия этих ионов на конкурентивное хлорирование.

3. Результаты исследований, проведенных ионами меди и соединениями, тормозящими процесс хлорирования, доказывают роль меди в реакции хлорирования.

РАЗЫСКИВАНИЕ НЕИЗВЕСТНЫХ БОЛЕЗНЕТВОРНЫХ АГЕНТОВ С ПОМОЩЬЮ ГЛАЗНОЙ РЕАКЦИИ МОРСКИХ СВИНОК

Б. РЕДЕИ, Ф. ЧИЗМАДИЯ

Глаза морских свинок, благодаря своей большой избирательной способности, выделяют из введенной в глаз смешанной культуры почти в чистой культуре бактерии, вызывающие на глазе кератоконъюнктивит. Хотя обладающие таким свойством штаммы и не все являются палочками Шига — как это полагалось до сих пор —, то на глазах морских свинок они все вызывают кератоконъюнктивит, а в человеческом кишечнике обуславливают подобные дизентерии изменения. (Авторы обнаружили даже два вызывающих кератоконъюнктивит штамма.) Значит, с помощью глазной реакции морских свинок можно, повидимому, объединить в патологическую единицу, штаммы вызывающие дизентерию и подобные дизентерии картины болезни.

На основе вышесказанного, авторы считают метод прививки глаз морских свинок смешанной культурой, предположительно содержащей болезнетворный агент, и пересев из воспалительного глаза, более эффективным методом, чем применяемые до сих пор методы, пригодным также для сознательного разыскания новых болезнетворных агентов. Предположение авторов подтверждается тем, что они с помощью этого метода уже неоднократно выявляли палочки Шига в таких случаях, когда другими методами не удалось их выделить. Они обнаружили своим методом также палочки Шига, которые до сих пор еще не встречались в Бенгрии, далее по всей вероятности новый болезнетворный штамм *E. coli*.

ИНДУКТИВНОЕ ПРОИЗВОДСТВО АМИЛАЗЫ МИЦЕЛИЕМ *PENICILLIUM CHRYSOGENUM*

И. ХОРВАТ, А. СЕНТИРМАИ, Ш. БАЮС и Э. ПАРРАГ

Производство амилазы промытым мицелием *Penicillium chrysogenum* имеет индуктивный характер. Присутствие крахмала необходимо только для индукции, процесс синтеза энзима совершается и без присутствия крахмала. Если удалить примененный для индукции крахмал до образования полной синтезирующей энзим системы, то наблюдается снижение производства энзима.

Глюкоза тормозит индуктивный синтез амилазы, но не влияет на производство энзима. Если добавляется глюкоза, до полного образования синтезирующей энзим системы, то наблюдается уменьшенный синтез энзима. Данные опыты показывают, что глюкоза — по словам Поллока — тормозит образование системы, „organizer” подобно фунгистатическим антибиотикам.

Выращиваемый на крахмальной питательной среде мицелий показывает в питательной среде низкий синтез энзима (0,5 Е/100 мл). Такие мицелии после промывания в присутствии крахмала снова могут быть индуцированы, подобно мицелиям, выращиваемым на глюкозе, хотя наблюдаемый на первоначальной питательной среде низкий синтез энзима не изменяется.

Процесс синтеза энзима можно тормозить 2,4-динитрофенолом, азидом натрия, цианидом калия, далее гидроксиламином, содержащем способную к реакции группу амина, как и гидразином фенила и эфирами различных аминокислот. Причиной тормозящего действия эфиров аминокислот предположительно является то, что способная к реакции группа аминов реагирует с активизированными аминокислотами подобно гидроксилламину.

ХАРАКТЕРИСТИКА ФАГОВ ПАЛОЧКИ СИБИРСКОЙ ЯЗВЫ

Ю. ЛАНТОШ, И. ВАРГА и Г. ИВАНОВИЧ

Авторы исследовали на 8 различных фагах свойства изолируемых из образцов почвы в большом количестве фагов, действующих на штаммы *Bac. anthracis*. Целям сопоставления служил W-фаг, полученный из лизогенного *Bac. cereus* (штамм W). Исследования распространялись на морфологию «бляшек», на чувствительность в отношении цитрата, на адсорбцию, на латентный период и на антигенную структуру фагов. Кроме того исследовалась также чувствительность фагов к ультрафиолетовому свету и теплу.

На основе упомянутых свойств, 9 фаговых штаммов можно отнести к трем группам. Основной разделения служит антигенная структура фагов, с которой их прочие свойства (теплочувствительность, чувствительность к ультрафиолетовому свету, к цитрату и т. д.) также можно ставить в параллель.

С одной частью фагов проводились также опыты по лизогенизации, которые, однако, либо не венчались успехом, либо получались в случае отдельных штаммов лабильные комплексы *Bac. anthracis* и фагов.

Авторы предлагают для обозначения фагов, распространенных в природе и обладающих в отношении штаммов *Bac. anthracis* специфической активностью, название «А-фаги» и признать прототипами последних изолированный Мак-Клейем W-фаг. Сообщенные исследования могут служить основой при систематизации и дальнейшем разделении А-Фагов.

ИНДУЦИРОВАННОЕ ПЕНИЦИЛЛИНОМ ОБРАЗОВАНИЕ ПРОТОПЛАСТОВ ИЗ КЛЕТОК *BAC. CEREUS* И *BAC. ANTHRACIS*

Я. ФЭЛДЕШ и К. МЕРЕТЕИ

В синтезирующем пенициллиназу штамме *Bac. cereus* NRRL 569 можно индуцировать при помощи пенициллина производство протопластов. В настоящей статье авторы излагают предпосылки и оптимальные условия индукции. Полученные протопласты не адсорбируют фагов и неспособны также к размножению. Полученные образования следует рассматривать настоящими протопластами.

ДИССОЦИАЦИЯ КУЛЬТУР *Sh. sonnei* НА ПИТАТЕЛЬНОЙ СРЕДЕ, СОДЕРЖАЩЕЙ ДЕЗОКСИХОЛЕВУЮ КИСЛОТУ

Б. ШЕРЕНЬ

1. Сообщается метод изготовления новой питательной среды — агар *D*.
2. На агаре *D* культуры *Sh. sonnei* в большой мере диссоциируют. Колонии фазы I и II, далее вариант *R* можно распознать на основании морфологических критериев колоний.
3. Путем культивирования на агаровых пластинках, изготовленных дрожжевой вытяжкой, как и на агаре *D* штаммов *Sh. sonnei* можно идентифицировать с большой надежностью.

ЭЛЕКТРОННОМИКРОСКОПИЧЕСКОЕ ИССЛЕДОВАНИЕ КОМПЛЕТНОГО И НЕКОМПЛЕТНОГО ВИРУСА ГРИППА

III. ИССЛЕДОВАНИЕ ТОНКОЙ СТРУКТУРЫ КОМПЛЕТНОГО ВИРУСА ГРИППА МЕТОДАМИ ДЕЗИНТЕГРАЦИИ И ПЕРЕВАРИВАНИЯ

И. ХОЛЛОШ

Очищенный комплетный вирус гриппа подвергался комбинированной дезинтеграции и перевариванию после нанесения на фиксированную формалином пленку. При переваривании трипсином обнаружилась известная форма кольца, которая разлагалась после обработки рибонуклеазой + трипсином. При переваривании трипсином после обработки эфиром наблюдались, кроме небольших клубочек, нитевидные образования, разложившиеся под действием рибонуклеазы + трипсина. Обработанный в суспензии эфиром вирус распадал на сфериды, среди которых удалось выявить небольшое количество коротких нитей.

РЕЗУЛЬТАТЫ ПРОТИВОСТОЛБНЯЧНЫХ ПРЕДОХРАНИТЕЛЬНЫХ ПРИВИВОК, ПРОВЕДЕННЫХ СРЕДИ ГРАЖДАНСКОГО НАСЕЛЕНИЯ

А. ПЕТРИЛЛА

Активная иммунизация против столбняка стала обязательной в Венгрии в 1953 году. С тех пор защитную прививку получили 6—11 месячные и шестилетние (с вакциной Ди-Пе-Те), далее 12 летние дети (с вакциной Ти-Те). Прививки были сделаны два раза с интервалом в 4 недели, и по истечении одного года проводилась ревакцинация. В 1958 году возрастную группу до 20 лет можно было считать практически привитой. Доля заболеваний столбняком составляла в период до обязательных прививок (1951—1952 гг.) в возрастной группе до 20 лет 9,2‰, а в 1958 году 1,7‰, значит, заболеваемость уменьшалась круглым счетом на 80%. Следовательно, прививки дали положительный результат, в частности если принимается во внимание, что 5—10% обязанных к вакцинации детей не получили предписанных прививок. Уменьшение заболеваний столбняком у новорожденных (моложе 1 года) следует приписать другим факторам, прежде всего тому, что роды произошли за это время из года в год во все большем соотношении в родильных домах, причем небольшая часть новорожденных относится к матерям, получившим защитную прививку в возрасте от 15—19 лет. Однако, не удалось определить сколько из заболевших лиц получило защитную прививку. 85% заболеваний столбняком возникло после таких повреждений, при которых больные не обращались к врачу, и следовательно для предохранения таких заболеваний пригодна только активная иммунизация.

ПОЛУЧЕНИЕ ХРОМОВАЛЬБУМИНА И ЕГО АНТИГЕННЫЕ СВОЙСТВА

Л. КЕСТЬЮШ, М. КАВАИ, Е. САБО и Л. ҚОЧАР

В ходе проведенных до сих пор опытов, авторам удалось разработать методику при помощи которой нативный овальбумин успешно подается хромированию. Полученный таким путем хромовальбумин и нативный овальбумин показали на основании анафилактических опытов и кольцевого осаждения поведение гомологичных антигенов. Согласно результатам агарового осаждения, нативный овальбумин содержит три антигенных фракции, и в иммунной сыворотке также можно выявить три антитела. Процесс хромирования делает эти три антигенных фракции однородными, и иммунная сыворотка также содержит только одно антитело, которое вступает в реакцию со всеми тремя фракциями нативного овальбумина.

ИССЛЕДОВАНИЕ ВАКЦИННОГО ШТАММА ПРОТИВ ТУБЕРКУЛЕЗА

Й. ВЕЙСФЕЙЛЕР и В. КАРАСЁВА

1. Обозначенный цифрой штамм 115 выделен из человеческого организма является ослабленным видоизмененным разновидностью туберкулезной палочки с фиксированной вирулентностью.

2. Штамм № 115 не изменял своей вирулентности для морских свинок в ходе пассирования на протяжении четырех лет.

3. Штамм № 115 после прививки морским свинкам размножается в организме в течение 4—5 месяцев, и обнаруживаются посевом еще 236 дней после прививки.

4. Опыты по иммунизации морских свинок показали, что штамм № 115 в случае подкожной и пероральной вакцинации вызывает более высокую степень иммунитета, чем штамм БЦЖ.

5. В отношении патогенных свойств для обезьян штамм № 115 показал также ослабленную вирулентность. Следовательно, его можно рассматривать как вакцинный штамм.

ИЗУЧЕНИЕ ВЗАИМООТНОШЕНИЙ МЕЖДУ ВИРУСАМИ КОКСАКИ И ПОЛИОМИЕЛИТА

IV. ДАЛЬНЕЙШИЕ НАБЛЮДЕНИЯ НА МОЛОДЫХ МЫШАХ АНТАГОНИЗМА МЕЖДУ ШТАММАМИ КОКСАКИ И ПОЛИОМИЕЛИТА ЛАНСИНГА

И. ДЕМЕК

Автор заразил внутричерепально молодых 9 дневных мышей вирусом коксаки В1 100000 краткой средней смертельной дозой а затем 10 дней спустя — вирусом полиомиелита (адаптированный к мышам штамм Лансинга) 10-краткой средней смертельной дозой. После заражения полиомиелитом было исследовано в пределах 10 дней содержание вирусов полиомиелита в нервной системе мышей. Вирус полиомиелита не размножался, ни в головном мозгу, ни в спинном мозгу мышей. На этих мышах, также как и на мышах, зараженных только вирусом коксаки В1, автор исследовал содержание вируса коксаки В1 и содержание антител в образцах центральной нервной системы в одинаковых с предыдущими опытами сроках. Несмотря на значительный иммунитет, вируса коксаки В1 удалось реизолировать из нервной системы на протяжении 18 дней после заражения.

На 43 день после заражения вирусом коксаки В1 после обработки кортизоном и прочих вмешательств, из совместной суспензии печени и поджелудочной железы удалось выявить вирус коксаки В1. Результаты доказывают, что длительная резистенция против полиомиелита, наблюдаемая в молодых мышах после заражения вирусом коксаки В1, обусловлена длительной стойкостью этого вируса.

ИССЛЕДОВАНИЕ РЕЗИСТЕНТНЫХ В ОТНОШЕНИИ БЕНЗОЙНОЙ КИСЛОТЫ ДРОЖЖЕВЫХ ГРИБОВ

М. ИНГРАМ

Из сока апельсина, консервированного бензоатом, изолировалось несколько штаммов дрожжевых грибов, подобных *Saccharomyces acidifaciens*. Было выявлено, что эти штаммы при величинах pH ниже 3, резистентны в отношении бензойной кислоты в концентрации 500 мг.л. Опыты по ассимиляции и дыханию показали, что эти дрожжи в незначительной мере способны разлагать бензойную кислоту. Согласно данным автора разложение происходит в следующих фазах: гидробензойная кислота, протокатеховая кислота, катехол и β -кететраадипиновая кислота.

ПАТОГИСТОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ ЗАРАЖЕННЫХ ДИЗЕНТЕРИЙНОЙ АМЕБОЙ (*ENTAMOEBА HISTOLYTICA*) КОШЕК

(Предварительное сообщение)

Т. БАКАЧ и М. ЯНКО

Авторы заразили внутри слепой кишки молодых кошек возбудителем амебной дизентерии (*Entamoeba histolytica*). Материал для прививки они получили из культуры кала детей с клинически и лабораторно установленной дизентерией. Зараженные животные на протяжении 6 месяцев находились под клиническим и лабораторным наблюдением. Серия опытов была закончена патогистологическим исследованием. Картину болезни амебной дизентерии характернее всего показала толстая кишка. В слизистой оболочке наблюдалась некротическая область, в подслизистом слое водянистое набухание и круглоклеточная инфильтрация, а в кишечных железах было обнаружено большое количество *Entamoeba histolytica*. В селезенке также удалось выявить присутствие *Entamoeba histolytica*. Авторы намерены продолжать свои опыты в этом направлении на большой серии опытов на животных.

СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ ВИДОВ *PSEUDOMONAS* ВЫЗЫВАЮЩИХ ЗАБОЛЕВАНИЕ КОНОПЛИ И ТУТОВОГО ДЕРЕВА

З. КЛЕМЕНТ и Л. ЛОВРЕКОВИЧ

Описанный в 1959 году в Югославии *Ps. cannabina* Satic et Dowson появился в 1957—1958 гг. также на венгерских районах выращивания конопли. Авторы проводили сравнительное исследование между *Ps. cannabina* и возбудителем заболевания тутового дерева *Ps. mori*. Исследования распространялись на сопоставление биохимических, фагосенситивных и серологических свойств.

Ps. cannabina отличается от штаммов *Ps. mori* в отношении патогенности, однако, по своим биохимическим свойствам он оказался близким к ним.

Из листьев конопли, зараженных *Ps. cannabina* в ходе исследований было выделено два до сих пор неизвестных фага. В то же время из зараженных побегов тутового дерева также удалось изолировать два фага. На основе спектра этих фагов выявилось, что оба изолированных из листьев конопли фага разлагали также бактерии тутового дерева. Отдельные фаги, изолированные из различных растений хозяев, оказались идентичными.

Из результатов серологических исследований выявилось, что антигенная структура *Ps. Cannabina* и *Ps. mori* весьма сходна, однако, она не совершенно идентична.

Резюмируя сказанное, можно установить, что возбудитель заболевания конопли в отношении биохимических свойств, фагосенситивности и антигенной структуры оказался весьма сходным с *Ps. mori*. Данное сходство такого большого размера, что выдвигается вопрос, следует ли рассматривать *Ps. cannabina* новым видом, или же только разновидностью формы *Ps. mori*.

ДВУСЛОЙНАЯ ПОЛИТРОПНАЯ ПИТАТЕЛЬНАЯ СРЕДА (СІG) ДЛЯ ИССЛЕДОВАНИЯ РАСХОДА ЦИТРАТА, ОБРАЗОВАНИЯ ИНДОЛА И РАЗЖИЖЕНИЯ ЖЕЛАТИНА

М. РОДЛЕР, Ш. ВЕРЕШ и П. ХЕДЫ

Авторы дают описание новой, обозначенной ими СІG, питательной среды для совместного выявления расхода цитрата, образования индола и разжижения желатина кишечными бактериями. В интересах быстрого и точного биохимического определения они считают необходимым применение по меньшей мере двух комплексных питательных сред, при помощи которых с уверенностью можно выявить характерные свойства кишечных бактерий. При рассмотрении 335 стандартных штаммов бактерий (храненных в коллекции штаммов авторов) они установили, что их новая политропная питательная среда в каждом случае дала ожидаемый результат. При помощи своей новой питательной среды и с политропной питательной средой Ногради—Родлера авторы исследовали изолированных из своего рутинного материала 1122 различных штамма кишечных бактерий и в связи с этим получили точную картину биохимических свойств данных штаммов.

ИССЛЕДОВАНИЕ ВИДОИЗМЕНЕНИЙ АНТИГЕННОЙ СТРУКТУРЫ ШТАММОВ ВИРУСА ГРИППА А₂

ДЬ. ТАКАТШИ и К. БАРБ

Исследовалось при помощи пробы перекрестного гемагглютинационного торможения и пробы истощения иммунной сыворотки изменение антигенной структуры штаммов, изолированных во время свирепствовавшей в 1957/58/59 годах в Венгрии эпидемии, вызванной вирусом гриппа типа А₂. На основании первой пробы, но в частности на основе последней реакции, удалось выявить такое же изменение антигенной структуры типа А₂, какое было описано в прежних исследованиях авторов в связи с типом А₁. Сушность варьирования заключается в прогрессивном превращении антигенной структуры.

ЗНАЧЕНИЕ БАКТЕРИОЛОГИЧЕСКИХ ИССЛЕДОВАНИЙ В ЗАБОЛЕВАНИИ БРОНХОЭКТАЗИЕЙ

Я. СИТА, Г. НЫРЕДИ и Ш. ФЕРЕНЦИ

1. Исследовалась бактериальная флора больных, страдающих бронхоэктазией далее чувствительность к антибиотикам выращенных бактерий. Выделения бронхов были взяты отсосом Ковача.

2. Из 281 выделения 195 больных выращивались около 450 штаммов, относящихся к 17 видам бактерий. Среди этих штаммов были выявлены в наибольшем количестве (39,1%) стрептококки (*Streptococcus viridans*) и 15,8% штаммов, относящихся к группе гонококков (*Neisseria*).

3. Было установлено, что бактериальная флора выделений бронхов страдающих бронхоэктазией больных неоднородна.

4. Самым успешным методом выращивания бактерий авторы считают, если выделения, наряду с рассевом непосредственно на плотную питательную среду, обогащаются также в бульоне и проводится еще анаэробный посев.

5. Резистенция выделенных штаммов в отношении антибиотиков совпадает с опытом, приобретенным на материале Государственного Института Гигиены, за исключением штамма *Streptococcus viridans*, который обладает повышенной резистенцией против стрептомицина, пенициллина и антибиотиков с широким спектром, причем встречались также штаммы, резистентные даже против эритромицина.

6. Среди 195 больных лечение привело в 86% случаев к хорошим результатам, у 3 больных улучшение прогрессировало только медленно, у 17 больных появился рецидив заболевания, а состояние 10 больных осталось без изменения.

7. В случае рецидива заболевания необходимо проводить повторное бактериологическое исследование выделений бронхов, ибо оно имеет первостепенное значение при прицельном лечении антибиотиками.

ИССЛЕДОВАНИЕ ЦИТОПАТОГЕННОГО ЭФФЕКТА АДЕНОВИРУСОВ НА ТКАНЕВЫХ КУЛЬТУРАХ ЧЕЛОВЕЧЕСКОГО АМНИОНА

И. НАС и М. ТОТ

Авторы исследовали на тканевых культурах человеческого амниона типичность цитопатогенного эффекта штаммов, относящихся к первым 7 типам аденовирусов. В соответствии с результатами прежних исследований удалось выявить дальнейшие отклонения в цитопатогенном эффекте различных типов. Разница проявлялась прежде всего в цитопатогенном эффекте штаммов типов 3 и 5. Остальные вирусные штаммы вызывали на тканевых культурах отчасти цитопатогенный эффект переходного характера, а отчасти они имели подобный типу 5 эффект. Вызванные вирусами дегенеративные изменения проявлялись, равным образом, как в клеточном ядре, так и в цитоплазме, и в соответствии с этим разницу между двумя типами вырождения удалось выявить как в ядре, так и в плазме.

ЭПИДЕМИЯ БОЛЕЗНИ БОРНГОЛЬМА (PLEURODYNIA) В 1958 ГОДУ В ВЕНГРИИ

И. ДЕМЕК, Е. МОЛЬНАР и О. РУДНАИ

От июня до октября 1958 года в Венгрии прошла эпидемия болезни Борнгольма, которая началась в западной части страны и постепенно распространялась дальше на восток. Согласно собранным авторами данным, врачи во всей стране диагностировали свыше 40 000 случаев заболевания болезнью Борнгольма. Заболеваемость была большей в возрастной группе ниже 15 лет. Во время эпидемии в двух родовспомогательных учреждениях регистрировались среди новорожденных в большом количестве заболевания, сопровождаемые симптомами миокарда или же менингоэнцефалита.

При помощи собранного во время эпидемии и после нее материала 965 исследований 720 лиц, страдавших различными заболеваниями, авторы проводили на тканевых культурах селезенки обезьян, или же сосуших мышей, опыты по выделению вируса, причем им удалось изолировать из 304 материалов 273 лиц 308 вирусных штаммов. Из выделенных штаммов 265 оказались вирусами коксаки типа В₃. Этот вирус был выявлен во время эпидемии из материала исследования больных, страдавших болезнью Борнгольма в 68%, в материале новорожденных, подозреваемых в заражении вирусом коксаки в 66%, а в материале больных, страдавших прочими болезнями — в 13% случаев.

Причинную роль этого типа вирусов в деле вызывания эпидемии Борнгольма подтверждали также опыты, поставленные для исследования содержания антител против вируса коксаки В₃ в образцах крови 151 больного, страдавшего болезнью Борнгольма, как и в образцах крови 149 больных, страдавших прочими болезнями. В период выздоровления сыворотка крови 86% больных, страдавших болезнью Борнгольма и сыворотка крови 19% больных, страдавших прочими болезнями, содержала нейтрализующих антител в разведении 1:5 или выше. В 113 случаях болезни Борнгольма были исследованы парные сыворотки, причем в 67 случаях удалось в течение болезни выявить повышение титра.

Вирус коксаки В₃ оказался болезнетворным агентом также в нескольких случаях асептического менингита и лихорадочного состояния. Можно предполагать, что этот тип вируса способен вызвать также герпетическую ангину. Клиницисты наблюдали у 4,6% исследованных больных, страдавших болезнью Борнгольма, также менингеальные симптомы, а у 13,8% также герпетическую ангину. Болезнь Борнгольма сопровождалась у детей скорее симптомами заболевания брюшины, а у взрослых скорее симптомами поражения грудной клетки. Число лейкоцитов осталось в 78%, но РОЭ только в 40% случаев заболеваний на нормальном уровне.

Одна часть больных, страдавших болезнью Борнгольма, выделила болезнетворного агента на протяжении длительного времени, однако, выделение вирусов у новорожденных не продолжалось свыше двух недель.

Между выделенными штаммами вируса коксаки типа В₃ проявлялись в отношении выращивания отклонения. Отдельные штаммы удалось выращивать только на тканевой культуре почек обезьян, а другие только на сосуших мышцах. Последние варианты встречались чаще в материале исследования новорожденных.

В годы эпидемии болезни Борнгольма заболеваемость полиомиелитом была в Венгрии ниже, чем в любое время за предшествующие 20 лет. Объяснение данного явления интерференцией вируса коксаки В и полиомиелита кажется очевидным.

СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ АНТИГЕННЫХ СВОЙСТВ И ХРАНИМОСТИ ПРИ КОМНАТНОЙ ТЕМПЕРАТУРЕ ВАКЦИНЫ ПО САЛКУ ВЕНГЕРСКОГО И ЗАРУБЕЖНОГО ПРОИЗВОДСТВА

П. ФЭЛЬДЕШ, И. СЕРИ и Ж. БАНОШ

Авторы исследовали антигенные свойства примененной в ходе эпидемии полиомиелита в 1959 году в Венгрии вакцины по Салку венгерского производства, после ее выдерживания при комнатной температуре в течение двух недель. Определение антигенных свойств (экстинкция) проводилось в опытах на морских свинках по Ш. Гарду. Полученные величины экстинкции по сравнению с храненными в леднике контролями не показали сигнификантной разницы. Согласно результатам опыта, хранение в течение двух недель при комнатной температуре не обуславливает измеряемого уменьшения антигенных свойств вакцины, значит оно допустимо. Одновременно были определены антигенные свойства храняемой согласно предписаниям (в леднике) вакцины зарубежного производства, примененной также в ходе эпидемии 1959 года в Венгрии. Величины экстинкции храняемой в леднике и при комнатной температуре вакцины венгерского производства, далее выдержанной в леднике зарубежной вакцины в отношении всех трех типов удовлетворяют определенным Ш. Гардом минимальным требованиям.

ИССЛЕДОВАНИЕ ВОСПРИИМЧИВОСТИ НЕПРЕРЫВНО КУЛЬТИВИРУЕМЫХ КЛЕТОЧНЫХ ШТАММОВ ПОЧЕК ОБЕЗЬЯН К ВИРУСАМ

М. ШИМОН и А. ЯНЧО

Авторы исследовали спектр действия вирусов на непрерывно культивируемый клеточный штамм с обозначением РМ почки обезьян. Клеточный штамм находится теперь в 40. пассаже и размножается точно так же как и первичные культуры клеток почки обезьян. Клеточный штамм РМ показал в 20., 30. и 38. пассажах регресс роста, по всей вероятности обусловленный факторами среды. Клеточная культура не преобразовывалась.

Сравнительные исследования лабораторных штаммов и клинического материала показали, что этот новый клеточный штамм почти так же успешно применим для изолирования кишечных вирусов, как первичная клеточная культура почки обезьян.

БЫСТРЫЙ МЕТОД ДЛЯ ИССЛЕДОВАНИЯ СБРАЖИВАНИЯ РАФИНОЗЫ ДРОЖЖЕВЫМИ ГРИБАМИ

Э. К. НОВАК

Автором был разработан метод для определения коэффициента сбраживания рафинозы дрожжевыми грибами, который точнее, быстрее и проще прежних способов. Сущность метода состоит в том, что из хроматограммы сброженного раствора рафинозы можно определить коэффициент сбраживания. Присутствие мелибиоза в сброженном растворе означает 1/3, присутствие галактозы — 2/3, в то время как отсутствие сахара — 3/3 брожение рафинозы.

Была разработана также новая техника для выявления сахара, при помощи которой без нагревания можно немедленно выявить сахар на хроматограммах.

При помощи этого метода 1 час после окончания брожения точно можно определить коэффициент сбраживания рафинозы.

Хроматографическим исследованием автору удалось выявить этапное расщепление и потребление рафинозы при сбраживании рафинозы дрожжевыми грибами *Saccharomyces carlsbergensis*.

ИССЛЕДОВАНИЯ ИНГИБИТОРОМ ВИРУСА ГРИППА, СВЯЗАННЫМ С ПРОИЗВОДНЫМИ АНИЛИНА

И. ХОЛЛОШ

Автор связывал прочищенный ингибитор Френсиса с производными анилина диазотической связью. Одна часть производных не изменила условий связывания и элюции ингибитора Френсиса вируса гриппа. Согласно другим авторам, однако, главным образом алкилированные производные, хотя и не изменили условий адсорбции, но элюция производила весьма медленно или же стала совершенно затяжной. Таким образом развивалось длительное конкурентное торможение не только в отношении рецептора-эритроцитов-вируса, но и в отношении рецептора-хорисаллантоидной оболочки-вируса.

В оплодотворенном яйце полчаса после заражения вирусом введенный в аллантоидную полость модифицированный ингибитор вызывал значительное торможение размножения.

Автор проводил на мышах только предварительные эксперименты. После заражения внутрибрюшное введение модифицированного ингибитора вызывало значительное торможение размножения; однако, внутривенное введение ингибитора одновременно с заражением, а затем внутрибрюшное введение каждые 12 часов оказались эффективными только на протяжении 24 часов.

Автор в дальнейшем при помощи модифицированного ингибитора желает исследовать роль клеточного рецептора в размножении вируса гриппа.

ТЕРРИТОРИАЛЬНЫЕ ОСОБЕННОСТИ ЭПИДЕМИЧЕСКОГО ГЕПАТИТА В ВЕНГРИИ ЗА 1952—57 ГГ.

К. ШОЛТ и И. ВЕДРЕШ

Эпидемический гепатит регистрируется в Венгрии с 1950 года. Прошло примерно 2 года до того времени, когда венгерские врачи сообщили случаи этой болезни, как случаи инфекционного заболевания.

Количество зарегистрированных случаев заболеваний эпидемическим гепатитом с 1952 года по 1956 год постепенно поднималось; с того времени держится на том же уровне.

Подъем заболеваемости по территории Венгрии за отчетный период не был одинаков. Неодинаковый подъем заболеваемости стал особенно выраженным с 1955 года, особенно на западной половине территории страны. Заболеваемость в Будапеште и в крупных городах (Дебрецен, Мишкольц, Печ, Сегед) значительно превышала заболеваемость окружающей их территории и среднюю заболеваемость страны.

Влияние проэпидемичивания населения гепатитом на изменение заболеваемости, относительно областей, за отчетный период установить было еще нельзя. Относительно вышеупомянутых крупных городов, в связи с изменениями заболеваемости, это уже можно было предполагать.

Заболеваемость и летальность гепатитом в 1957 году были самые высокие на западной половине территории страны, самые низкие на Большой Венгерской Низменности. Причина этого явления неизвестна, изучение его может явиться предметом интересных клинико-эпидемиологических исследований.

Изменение заболеваемости не было одинаково в разномасштабных местностях. Села и города страны мы делили по количеству населения на 8 групп, девятой считался город Будапешт. Анализируя заболеваемость в этих группах с 1952 года по 1957 год мы нашли постепенный подъем; только в городах с населением выше 50 000 можно было обнаружить снижение заболеваемости в 1957 году. В населенных пунктах с населением до 10 000 — средние цифры заболеваемости эпидемическим гепатитом за 1952—57 гг. существенно не отличались друг от друга. За это же время в населенных пунктах с населением от 10 000 до 150 000 человек нам удалось найти постепенный подъем заболеваемости. Заболеваемость эпидемическим гепатитом с 1952 года по 1957 год в населенных пунктах с разным количеством населения показывает образ постепенного уравнивания. Разница между группами с самой высокой и самой низкой заболеваемостью снизилась с пятикратной на двухкратную. В этом, по всей вероятности, между прочими факторами играет роль и центрифугальное распространение эпидемического гепатита из городов в деревни.

Изучая возрастное распределение заболевших гепатитом на некоторых территориях — с самой высокой и самой низкой заболеваемостью — совпадая с данными литературы выяснилось, что соотношение среди заболевших в возрасте от 0 до 14 лет выше на территориях с высокой заболеваемостью, чем территориях с низкой заболеваемостью.

По данным литературы можно найти взаимосвязь между уровнем заболеваемости гепатитом и благоустройством населенного пункта.

Был произведен анализ заболеваемости эпидемическим гепатитом за 1954—57 гг. неканализованных и канализованных городов с населением от 20 000 до 100 000 человек. Выяснилось, что — не совпадая с данными литературы — заболеваемость эпидемическим гепатитом была выше в канализованных городах, чем в неканализованных. Проанализировав также данные о заболеваемости брюшным тифом — мы получили подобные результаты. Из этих данных можно предполагать, что в настоящее время в распространении этих двух кишечных инфекций решающую роль играет бытовой контакт.

Нам не удалось найти взаимосвязь между количеством врачей данной территории, густотой населения, процентом госпитализации больных и заболеваемостью территории.

О СИСТЕМЕ ЛИПАЗЫ ЛЕПТОСПИР

1. ИССЛЕДОВАНИЕ АКТИВНОСТИ ТРИБУТИРИНАЗЫ

Л. БЕРТОК и Ф. КЕМЕНЕШ

Авторы исследовали методом Комфорта производство липазы патогенными и сапрофитными лептоспирами. Удалось выявить, что штаммы, способные расщеплять жиры животного происхождения, способны также гидролизировать растительные масла и трибутирин. Притом сапрофитные лептоспиры способны гидролизировать жиры животного происхождения в меньшей, а трибутирин в большей степени. В ходе исследований авторы получили данные относительно появления липазы лептоспир (трибутириназы) в культурах, о их отношении к гемотоксину, о их термической инактивируемости, далее о их фильтруемости и недифференцируемости.

В дальнейшем авторы сравнивали активность трибутириназы имеющихся в их коллекции сапрофитных и патогенных лептоспир, и выявили значительные отклонения между липазной системой патогенных и сапрофитных лептоспир. Далее они исследовали скорость реакции липазы лептоспир, как и возможность воздействовать на последнюю парализаторами (атоксил, неосальварсан и фторид натрия и т. д.).

В заключение авторы устанавливают, что подробное знание биологических свойств лептоспир предоставляет лучшую основу объяснения развития лептоспирозов, их эпидемиологии и прочих наблюдений, связанных с этим заболеванием, чем прежние подробные исследования антигенной структуры лептоспир.

ЭКСПЕРИМЕНТАЛЬНЫЕ ДАННЫЕ О МИКРОБИОЛОГИИ РАССОЛА

Г. БИРО

Автор проследил при помощи микробиологических исследований за 20-дневным процессом просола ветчины. Общее количество бактерий снизилось во время просола с неравномерными колебаниями от величины 10^8 до 10^6 . Кишечную палочку в рассоле удалось выявить только до восьмого дня просола.

Среди изолированных бактериальных штаммов два штамма микрококков проявляли наибольшую способность расщеплять нитрат. При исследовании этих двух штаммов было установлено, что в питательной среде с большим содержанием соли денитрификационная деятельность бактерий повышалась, а при большем процентном содержании соли (свыше 22%) питательной среды с содержанием сахарозы они обладали повышенной солеустойчивостью; автор приводит это явление в связь с образованием слизи микрококками.

Исследованные штаммы кишечной палочки в бульоне с 13 процентной концентрацией соли больше не вырастали. При микробиологической оценке рассолов присутствие кишечной палочки указывает на неправильное проведение просола, прежде всего на ненормальное разжижение рассола.

ИССЛЕДОВАНИЯ ФАГОВ ШТАММА *CL. PERFRINGENS* II. ИССЛЕДОВАНИЕ ЛАТЕНТНОГО ПЕРИОДА РАЗМНОЖЕНИЯ ФАГОВ

ДЬ. ГАШПАР

1. Была установлена одноступенчатая кривая размножения фага штамма *Cl. perfringens* 808/3a. В течение латентного периода на кривой наблюдается медленный подъем.

2. В латентной фазе размножение фагов исследовалось методом торможения созревания фагов. Сущность метода сводится к тому, что созревание фагов задерживается в различных сроках, путем помещения системы в аэробные условия.

3. В течение латентной фазы можно различать две стадии.

а) В первой стадии не появляются новых частиц, и вследствие аэрации культуры титр фага в этой стадии выражено снижается.

б) Во второй стадии после прибл. 35 мин. анаэробной инкубации начинается освобождение новых инфективных частиц, и после этого освобождение частиц продолжается также в аэробных условиях.

4. В объяснение повышения титра фагов в течение латентной фазы автор полагает, что вследствие продувания азота цепи *Cl. perfringens* разрываются и адсорбированные на одной и той же цепи фаговые частицы образуют на ставших самостоятельными бактерияльными индивидуумами особые бляшки.

5. Задерживающее созревание фагов действие анаэробной среды направляется на создавшийся комплекс бактерия-фаг, а не влияет в отдельности на эти компоненты.

ОТПЕЧАТКИ КОЛОНИЙ ШТАММОВ *Sh. SONNEI* НА АГАРНОЙ ПИТАТЕЛЬНОЙ СРЕДЕ

Б. ШЕРЕНЬ

После удаления колоний штаммов *Sh. sonnei* изолированно культивированных на агаровой пластинке, изготовленной дрожжевой вытяжкой, на питательной среде видны отражающие структуру колоний образования, названные автором отпечатками колоний. Отпечатки колоний, в которых можно выявить антигены исключительно только II фазы, проявляются в виде круглых, беловатых, сильно мутных образований. В отпечатках звездчатых колоний наблюдается звездчатый рисунок. Если колонии слагаются особами содержащими исключительно антигены I фазы, то после удаления колоний на питательной среде не остается отпечатка. Вышеизложенное явление до известной степени можно использовать для определения содержания антигенов в культурах *Sh. sonnei*. Согласно результатам исследований отпечатки возникают в результате того, что в колониях и частях колоний, относящихся к II фазе образуются агломераты, прилипающие к агару.

ИССЛЕДОВАНИЕ ЗАРАЖЕННОСТИ СТАФИЛОКОККАМИ В БОЛЬНИЦАХ МЕТОДОМ ОПРЕДЕЛЕНИЯ ФАГОТИПА

Х. МИЛЬХ, М. ЭРШИ и М. БОГАРДИ

При исследовании зараженности стафилококками больных и персонала больниц на основании 1782 массовых обследований, 10% больных оказалось при приеме в больницу положительными, а в течение пребывания в больнице 71% больных стал положительным. 60% персонала оказалось носителями стафилококков.

На основании результатов определения фаготипа отдельные фаговые группы стафилококков в порядке и встречаемости следующие: I., смешанная, III. и II. группы. Встречаемость отдельных фаговых групп оказалась у больных и у персонала различной. Штаммы стафилококков, относящихся к группе II. встречались у персонала в значительно большем проценте, а штаммы фаговой группы III. были обнаружены в большем количестве у больных.

При помощи определения фаготипа прослеживались также пути распространения отдельных штаммов. Способность к распространению оказалась у штаммов II. группы меньшей, чем у других групп. Взаимное заражение больных или их заражение от персонала оказались почти одинаковыми.

Фаготипы были у 55% исследованных лиц постоянными. Суперинфекции наблюдаются чаще у больных, чем у здоровых взрослых.

При исследовании связи между устойчивостью к антибиотикам и фаготипами наибольшей устойчивостью обладала фаговая группа III., в отношении всех исследованных антибиотиков. Исследовалась далее связь между изменением антибиогаммы и образованием фаготипа. На основании серийных исследований одних и тех же лиц было установлено, что изменения фаготипа и чувствительности к антибиотикам встречаются также независимо друг от друга. В случае появления штамма, относящегося к новой фазовой группе, чувствительность к антибиотикам изменяется сигнификантно чаще, чем в случае штамма относящегося к другому типу в пределах одной и той же фаговой группы.

ИССЛЕДОВАНИЕ СОСТАВА АМИНОКИСЛОТ В КЛЕТОЧНОЙ СТЕНКЕ ШТАММОВ *E. coli* O:111 С РАЗЛИЧНОЙ ЧУВСТВИТЕЛЬНОСТЬЮ К АНТИБИОТИКАМ

Л. ВАЦИ, М. ФОДОР, А. РЕТИ и И. ХОЛЛОШ

Авторы исследовали состав аминокислот клеточной стенки *in vivo* чувствительных и *in vitro* резистентных к антибиотикам штаммов *E. coli* O:111 на препаратах клеточной стенки, изготовленных различными способами и контролируемых электронномикроскопическими съемками.

В клеточной стенке штаммов *E. coli* O:111 с различной чувствительностью к антибиотикам было выявлено 17 аминокислот.

В клеточной стенке удалось выявить в наибольшем количестве глютаминовую кислоту, аланин, аспарагиновую кислоту и леуцины, далее диамино-пимелиновую кислоту.

Относительно количества аминокислот, относящихся к основным структурным элементам стенки между чувствительными и резистентными клетками не наблюдалось существенной разницы.

Разница содержания диаминопимелиновой кислоты, выявляемая в препаратах клеточной стенки, изготовленных различными способами препарирования из чувствительных и резистентных бактерий, указывает на отклоняющееся структурное строение поверхности клеток с различными биологическими свойствами.

ДАННЫЕ К ИЗГОТОВЛЕНИЮ ЧЕЛОВЕЧЕСКИХ ПЕРВИЧНЫХ АМНИОТИЧЕСКИХ КЛЕТОЧНЫХ КУЛЬТУР

И. БЕЛАДИ, Э. КУКАН, И. МЕЧ и Э. СЕЛЛЕШИ

В ходе трипсинизирования 252 амниотических оболочек авторы разработали метод изготовления первичных амниотических клеточных культур. Они сравнивали результаты трипсинизирования, проведенного при температуре 33 °C и 37 °C, и считают трипсинизирование при температуре 37 °C лучшим способом. Далее авторы исследовали, каким образом можно использовать морфологическую картину, коэффициент окрашивания трипановой синькой при оценке пригодности клеток для изготовления тканевых культур, как и влияния различных сывороток. Они считают предварительную установку нескольких культур в пробирках наиболее соответствующим методом для решения того вопроса, применимы ли клетки, полученные трипсинизированием, для изготовления тканевых культур. До оценки этих культур остальные клетки хранятся в питательной среде с содержанием глютамина. Хранение не влияет на жизнеспособность клеток. Применением этого метода отпадает много излишней работы.

БЫСТРОЕ БИОХИМИЧЕСКОЕ ИЗОЛИРОВАНИЕ КИШЕЧНЫХ БАКТЕРИЙ АГАРНОЙ ДИФФУЗИЕЙ И МИКРОМЕТОДАМИ

Б. ЛАНЬИ и М. АДАМ

Авторы разработали метод для быстрой и простой идентификации кишечных бактерий в ходе повседневной работы. Исследуемая колония засевается в жидкую питательную среду, комбинированную из мочевины и индола, а затем, после инкубации в течение нескольких часов, культура наносится на дезоксихолат-феноловокрасную агаровую пластинку. Расщепление декстрозы, маннита, лактозы, инозита, сахарозы и дульцита исследуется на этой пластинке, размещением бумажных полосок, пропитанных вышеприведенными веществами. Эта же пластинка служит для определения образования газа и H_2S , далее для определения реакции с метилротом. Дается описание простой капельной пробы для проведения реакции триптофановой деаминазы. Дезоксихолат-фенол-красный агар эффективно задерживает рост бактерий, являющихся загрязнением.

Авторы сравнивали полученную новыми методом реакцию 479 кишечных бактерий со стандартным методом. Результаты показали почти 100%-ное совпадение. Большое отклонение наблюдалось при реакции с метилротом (6.7%). Полезность нового быстрого метода доказывается тем, что в течение суток удалось определить соответствующую биохимическую группу в 95,2% из дальнейших 649 штаммов кишечных бактерий, изолированных в ходе исследования материала нефекального происхождения.

ПРОФИЛАКТИЧЕСКАЯ ВАКЦИНАЦИЯ ПРОТИВ ПОЛИОМИЭЛИТА В ВЕНГРИИ

Т. БАКАЧ

На основании оценки применения вакцины по Салку в Венгрии можно установить, что эта вакцина эффективна. Ее недостатком является, однако, что созданная ею иммунизация не продолжительна. Полученная в результате прививки иммунность уже на второй год сильно снижается. Даже оптимальная вакцинация по Салку не защищает полностью подвергнутую опасности популяцию. Эпидемиологическими исследованиями было доказано, что даже среди оптимально, то есть три раза привитой популяции может иметь место значительная заболеваемость. Течение болезни у привитых лиц, как правило, менее тяжелое, чем у непривитых. Вакцинация по Салку не препятствует ни энтеральному размножению вируса, ни рассеянию вируса среди популяции, и следовательно возможность эпидемий полиомиелита в дальнейшем также налично.

На основании всего сказанного санитарные органы пришли к тому решению, что в будущем в Венгрии будет применена вакцинация разведенной вакциной по Сабину.

СРАВНИТЕЛЬНОЕ ЭЛЕКТРОННОМИКРОСКОПИЧЕСКОЕ ИССЛЕДОВАНИЕ ФАГОВ, ДЕЙСТВУЮЩИХ НА МИКРОБАКТЕРИИ

Ф. ГУБА и Э. ВАНДРА

Авторами были исследованы морфологические данные 5 видов микробактериофагов (*M. friburgensis*, *M. phlei*, *M. rabinowitsch*, *M. smegmatis*, *M. butyricum*) и установлено, что они отличаются друг от друга как по размерам головки, так и по размерам хвоста.

ДАННЫЕ О ЧАСТОТЕ СЕРОТИПА E. COLI O:125 (VAR. CANIONI)

ДЬ. УЙВАРИ и Г. ПАЛЛ

Авторы сообщают о небольшой больничной эпидемии, вызванной редким, энтеропатогенным штаммом *E. coli*. В результате проведенных с января 1959 года до июня 1959 года систематических бактериальных исследований кала больных грудных детей и детей было в 52 случаях установлено наличие штамма *Coli dyspepsiae* O:125 с антигенным знаком B:15. Этот штамм, изолирование которого раньше ни в одном случае не уда-

лось, попал в больницу с калом грудного ребенка, принятого в отделение больницы с симптомами тяжелого энтероколита. В течение последующих 6 месяцев удалось изолировать этот штамм из кала 52 больных в возрасте от 1 месяца до 4 лет. У 30 из 52 больных с положительным калом наблюдались энтеральные симптомы, которые в 18 случаях соответствовали слабому, в 9 случаях среднему тяжелому, а в 3 случаях тяжелому энтероколиту. (57.7%), 22 больных оказалось с точки зрения энтероколита свободными от симптомов бацилловыделителями (42.3%).

Авторы подчеркивают значение снижения устойчивости организма, которое имеет место в связи с внекишечными патологическими процессами (грипп, гриппозные осложнения) в развитии клинических симптомов энтеральной картины болезни, учитывая особенно типы штамма *Coli dyspersiae*. Кроме эффективной антибиотической терапии (неомицин, хлостацилин), примененной на основе чувствительности к антибиотикам *in vitro* авторы обращают внимание на важность вливания глюкозы и рингеровского раствора, плазмы, цельной крови, гамма-глобулинов и введения поливитаминов.

В связи со своими эпидемиологическими наблюдениями авторы указывают на некоторые внешние факторы, которым они приписывают роль в возникновении больничной эпидемии.

Подробно излагаются морфологические, биохимические и серологические особенности изолированных штаммов, как и их чувствительность к антибиотикам.

На основании количественных и качественных исследований чувствительности к антибиотикам, эффективными оказались неомицин, хлороцид, стрептомицин или же тетрацилин.

На основании оценки результатов исследований авторы приходят к тому заключению, что серотип В:15 является условнопатогенной формой *E. coli* O:125 для детей грудного возраста.

ИЗОЛИРОВАНИЕ НЕКОТОРЫХ КОМПОНЕНТОВ *MYCOBACTERIUM TUBERCULOSIS* И ИССЛЕДОВАНИЕ ИХ СВОЙСТВ

Я. ФЁЛДЕШ

Автором были разработаны новые методы изготовления препаратов для изолирования макромолекулярных компонентов возбудителя туберкулеза (*Mycobacterium tuberculosis*). Одна часть липоидов и липопротеинов удаляется ацетоном и фенолом, а затем при помощи буферного раствора, содержащего метиловый спирт проводится извлечение полисахаридных веществ. После этого «Bacterial residue» растворяется путем щелочного гидролиза и из раствора проводится фракционированное осаждение комплексных соединений липопротеинового характера и полисахаридов. Автор дает описание некоторых типичных данных химического анализа изолированных и очищенных соединений. С осажденными сульфатом аммония липопротеиновыми фракциями связана в постепенно повышающемся соотношении ДНК. В вытяжках полисахаридного характера были обнаружены нуклеиновая кислота или же пентоза, количество которых менялось в зависимости от соотношения объема осаждающего спирта. Два объема спирта осаждают полисахариды, в которых содержание пентозы меньше, а содержание нуклеиновой кислоты больше, в то время как в полисахаридах, осаждаемых пятью объемами спирта количество пентозы больше, а соотношение нуклеиновых кислот значительно меньше.

ПРИРОДА СПЕЦИФИЧЕСКИХ ПОЛИСАХАРИДОВ ТУБЕРКУЛЕЗНОЙ ПАЛОЧКИ III.

Я. ФЁЛДЕШ

Автор характеризует два изолированных из туберкулезной палочки полисахарида различного типа химическими, физикохимическими и серологическими методами и пытается определить их место в структуре клетки.

Изолированные полисахариды серологически активны и гомогенны. На основании серологической активности они чувствительны к кислотам и устойчивы к щелочам. В ультрацентрифуге оба типа полисахаридов оказались гетеродисперсными. Согласно

химическим исследованиям вытяжка П I не гомогенна, в ней содержится большое количество нуклеиновых кислот (ДНК и РНК). С полисахаридом П II связано меньшее количество ДНК. Фракции со знаком *a*- и *b*- полисахаридов отличаются друг от друга в отношении содержания пентозы, разница двух или трех-кратная. Оба полисахарида содержат аминокислоты и аminosахары, указывающие на их происхождение из клеточной стенки.

Автор полагает, что вытяжка П I принимает участие в построении внешнего слоя клеточной стенки, а полисахарид типа П II участвует в создании внутреннего слоя.

ХИМИЧЕСКОЕ И СЕРОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ ПРОТЕИНОВ *MYCOBACTERIUM TUBERCULOSIS*

Я. ФЁЛДЕШ

В прежних работах автор сообщил о фракционированном изолировании макромолекулярных компонентов клетки туберкулезной палочки, далее об исследовании изолированных полисахаридов. Настоящая статья занимается изолированными веществами протеинового характера. Автор установил, что

1. протеиновые фракции II—V представляют собой химически сложные макромолекулы: протеины связаны с полисахаридами и с нуклеиновой кислотой. Эти компоненты встречаются в отдельных протеиновых фракциях в постепенно повышающемся количестве. Вытяжки содержат также липоиды.

2. На основании хроматографического анализа протеины построены из по меньшей мере 12 различных аминокислот, причем полисахаридная часть состоит из арабинозы, глюкозы и галактозы. Содержание нуклеиновой кислоты следует рассматривать исключительно как ДНК.

3. Вытяжки оказались серологически активными: они осаждают иммунную сыворотку кроликов, и в тесте торможения геммагглютинации по Миллбрук—Дюбо связывают антитела, но не сенсibiliзируют эритроцитов. Размер серологической активности соразмерен содержанию полисахаридов в вытяжках.

4. На основании агар-диффузионного метода вытяжки следует рассматривать гомогенными, они дают лишь один преципитационный пучок. На основе ультрацентрифугального анализа находящиеся в вытяжках макромолекулы образуют гетеродисперсную систему.

5. Изолированные вещества протеинового характера принимают участие в построении внутреннего вещества протоплазмы.

ИССЛЕДОВАНИЕ ЛЕПТОСПИРОЗНОЙ ЭПИДЕМИИ В ОКРЕСТНОСТИ С. ТАПИО

М. ФЮЗИ и Й. КИСЕЛ

Летом 1954 года в окрестности с. Тапиосентмартон прошла лептоспирозная эпидемия. Случаи заболеваний наблюдались отчасти среди трудящихся местного государственного хозяйства, а отчасти в кругу деревенских жителей, купавшихся наволе или работавших на полях в период до эпидемии. Случаи лептоспирозов носили доброкачественный характер, случаев с летальным исходом не было. Из 10 серологически доказанных случаев заболеваний в 4 случаях возбудителями инфекции оказались *L. pomona* в дальнейших 4 случаях — *L. grippo-typhosa* и в 2 случаях — *L. hyos*.

В целях исследования источников заражений летом 1955 года проводились массовые серологические исследования домашних животных. Методом реакции агглютинации-лизиса исследовалась сыворотка крови 50 свиней, 40 голов крупного рогатого скота и 42 овец, причем применялись следующие серотипы: *L. icterohaemorrhagiae*, *L. canicola*, *L. ballum*, *L. australis A*, *L. australis B*., *L. pomona*, *L. grippo-typhosa*, *L. hebdomadis*, *L. sejroe*, *L. bataviae*, *L. hyos*.

Среди свиней 42 (84%), среди крупного рогатого скота 13 (32%) оказались сероположительными, в то время как среди овец всего только в 4 случаях (9,5%) был получен положительный результат. Сыворотки крови свиней реагировали с типами *L. pomona*, *L. hyos* и *L. sejroe*, в то время как среди крупного рогатого скота удалось выявить зараженность *L. pomona*, *L. sejroe* и *L. grippo-typhosa*. В сыворотках овец обнаруживались только низкие титры *L. icterohaemorrhagiae*.

Эпидемиологические наблюдения и результаты исследований указывают на то, что частые заболевания возникали в результате почти одновременных заражений из различных источников (свиньи, крупный рогатый скот, полевые грызуны).

ИССЛЕДОВАНИЯ СПОСОБА ИЗГОТОВЛЕНИЯ ПИТАТЕЛЬНЫХ СРЕД С СОДЕРЖАНИЕМ ГЛЮКОЗЫ. КУЛЬТИВИРОВАНИЕ *RHIZOBIUM MELILOTI* В ФЕРМЕНТОРАХ

Б. ЙОХАН, Й. СЮЧ-САБО и А. САБО

Авторы встряхивали *Rhizobium meliloti* в колбах и затем культивировали их в ферменторах. Питательная среда состояла из гидролизованного кислотой казеина, вытяжки пекарских дрожжей, картофельного сахара и определенных неорганических солей, — в других случаях также из неорганических солей, кукурузного повидла, автолизата пивных дрожжей и картофельного сахара. Когда составные части питательной среды стерилизовались совместно обычным способом при высокой температуре, в отдельных случаях в этой питательной среде не наблюдалось роста. Если же обособленно стерилизованный сахар добавлялся после совместной стерилизации остального материала, то *Rhizobium meliloti* показал более слабый рост. Значит *Rhizobium meliloti* требует вещества, возникающего во время термообработки сахара вместе с остальными составными частями питательной среды. Если же термообработка (стерилизация) проводится сверх определенного времени или температуры, то рост или слабее, или же совершенно не имеет места. Авторы определили экспериментами допустимый размер термообработки. Если термообработка превышает оптимальный размер, то на такой питательной среде наблюдается продление латентной фазы роста. В случае дальнейшего повышения степени стерилизации, рост совершенно не имеет места. Стерилизованная сверх оптимального времени питательная среда принимает темно бурый цвет. Интенсивность цвета соразмерна степени термообработки. Интенсивность бурого цвета определяется фотометром и выражается показателем экстинкции. Это побурение соответствует описанной Майардом реакции. В случае стерильного хранения при комнатной температуре эти питательные среды стали постепенно все более бурыми, их показатель экстинкции повышался, а по истечении определенного времени они оказались — при высоком показателе экстинкции — совершенно непригодными для культивирования *Rhizobium meliloti*. Прибл. 20 мин. стерилизация при температуре в 120 °C вполне достаточна и дает хороший рост. Медленное нагревание, а особенно охлаждение ферментора приводит к повышению показателя экстинкции.

СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ ЭФФЕКТИВНОСТИ РАЗЛИЧНЫХ СТАНДАРТНЫХ ВАКЦИН ПРОТИВ КОКЛЮША

И. ЙОО, Ж. ПУСТАИ и В. П. ЮХАС

Излагаются исследования авторов, в ходе которых было приготовлено несколько лиофилизированных стандартных вакцин против коклюша. При помощи метода внутричерепной активной защиты мышей эти вакцины были сопоставлены зарубежным (США, английским и международным) вакцинам против коклюша. Для статистической оценки результатов применялся метод Уйлсона—Уорчестера. Из результатов экспериментов можно сделать следующие заключения:

1. Между эффективностью американской (USV) английской (BV) и венгерской (HuV1) стандартными вакцинами не отмечалось значительно разницы.
2. Защитная ценность венгерских HuV2. и HuV3. стандартных вакцин повидному сильнее (прибл. 1,5-кратно) эффективности USV, BV и HuV1 стандартных вакцин, хотя и не сигнификантна.
3. Защитное действие международной стандартной вакцины (I V) сигнификантно выше эффективности остальных стандартных вакцин.
4. Между результатами параллельного определения эффективности одной и той же вакцины в одном и том же опыте не было выявлено сигнификантной разницы.

5. Согласно наблюдениям авторов активной внутрицеребральной пробой защиты мышей в 2—3 повторных исследованиях удовлетворительным образом можно определить для практики защитную ценность вакцины.

6. Результаты исследований подтверждали наблюдения других авторов, согласно которым приготовленная соответствующим образом вакцина против коклюша в течение нескольких лет неизменно сохраняет свою эффективность.

В заключение авторы сравнивают свои результаты с подобными исследованиями других авторов и обсуждают некоторые проблемы метода активной защиты мышей.

РАСПРЕДЕЛЕНИЕ СЕРОТИПОВ *SHIGELLA FLEXNERI* В ВЕНГРИИ В ПЕРИОД ОТ 1945—1958 ГГ.

А. ВЕРТЕНЬИ

1. За годы от 1945—1958 автор проводил определение типа 2156 штаммов *Sh. flexneri*. Чаще всего встречался тип 2а (65,8%), а затем тип 3 (18,6%), остальные типы были обнаружены в количестве 0,1—2,8%. В отношении динамизма распределения типов кроме повышающегося преобладания типа 2а, число зараженностей типом 3 показывает повышение, в то время как встречаемость типов 1b и 6 имела снижающую тенденцию.

2. Ответственными за эпидемии практически следует считать только типы 2а и 3.

3. Географическое распределение типов *Sh. flexneri* в сущности совпадает со средним распределением, однако, в южных и восточных областях страны наблюдается большая встречаемость типа 3, в то время как на отдельных территориях можно было установить относительное, преобладание определенных местных типов (4а, 6).

4. На основе исследования хронических бацилловыделителей можно установить, что в этой клинической картине можно обнаружить практически все типы, и что в соотношении встречаемости отдельных типов только вариант Y показывает сдвиг по сравнению со средними данными, что указывает на характер минусварианта этого типа.

5. Полученные данные были сопоставлены с подобными исследованиями других авторов, причем автор указывает на значение определения серотипов.

ИЗОЛИРОВАНИЕ НОВЫХ ФАГОВ СТАФИЛОКОККОВ И ИХ ИСПОЛЬЗОВАНИЕ ДЛЯ ОПРЕДЕЛЕНИЯ ФАГОТИПОВ

Х. МИЛЬХ

Из лизогенных стафилококковых штаммов были изолированы фаги для идентификации не поддающихся типизации штаммов. Активность растворения новых фагов была исследована на 865 штаммах. При помощи новых фагов удалось идентифицировать 46% не типизируемых штаммов.

Автором был разработан новый метод для приготовления антифаговой сыворотки и при помощи полученных антифаговых сывороток он определил группы, в которые можно отнести новые фаги.

Наивысшую резистентность к антибиотикам показали идентифицируемые изолированными автором фагами стафилококковые штаммы. Высокой резистентностью и антибиотикам обладали еще не типизируемые и относящиеся к III. фаговой группе штаммы.

В больничном отделении новорожденных автором была исследована эпидемия, вызванная стафилококками. В 60% случаев удалось выявить фаготип со знаком 81(304) (международное обозначение тип 81), а в 23,3% случаев — фаготип с обозначением 756/950 (основными фагами его нельзя типизировать). Автор наблюдал, что относящиеся к фаготипу 756/950 штаммы обладают повышенной патогенностью и их резистентность к антибиотикам весьма высокая.

ЭПИДЕМИЯ ПОЛИОМИЕЛИТА В 1959 ГОДУ В ВЕНГРИИ

О. РУДНАИ

1. В 1959 году в Венгрии прошла эпидемия, вызванная вирусом 1-го типа, при котором было зарегистрировано 1830 случаев заболеваний. Заболеваемость выражалась цифрой $18,3\text{‰}$. Более высокая заболеваемость наблюдалась только в одном единственном году, а именно в 1957 г.

2. Эпидемия началась в столице и отсюда распространялась по всей стране, так что заболеваемость оставалась ниже 10‰ только в 4 областях страны. Заболеваемость была выше всего в области Пешт ($30,9\text{‰}$).

3. Государственная кривая эпидемии 1959 года начала подниматься в июне и достигала своего максимума в августе. В Будапеште максимальное число заболеваний зарегистрировалось в июле. Кривая эпидемии — вероятно под влиянием проведенных во время эпидемии предохранительных прививок вакциной по Салку — показала более крутое падение, чем во время предыдущих эпидемий.

4. Возрастноспецифическая заболеваемость в 1959 году сильно изменилась. В то время как в предыдущих годах выше всего была заболеваемость годовых детей, в 1959 году заболеваемость грудных детей превысила заболеваемость всех остальных возрастных групп. В то же время заболеваемость была в возрастной группе от 1—2 лет ниже, чем в 1952—57 гг. Вышеупомянутое изменение распределения заболеваемости по возрастным группам обуславливалось проведенными с 1957 года профилактическими прививками.

5. Летальность была весьма низкой, всего только $2,9\text{‰}$.

Летальность привитых составляла $1,8\text{‰}$, а непривитых — $4,6\text{‰}$.

Следовательно понижение средней летальности наступило на действие вакцинации.

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